Re-analysis of public genetic data reveals a rare X-chromosomal variant associated with type 2 diabetes

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Published in:
Nature communications

DOI:
10.1038/s41467-017-02380-9

Publication date:
2018

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):

Download date: 17. aug., 2018
Re-analysis of public genetic data reveals a rare X-chromosomal variant associated with type 2 diabetes

Sílvia Bonàs-Guarch et al.

The reanalysis of existing GWAS data represents a powerful and cost-effective opportunity to gain insights into the genetics of complex diseases. By reanalyzing publicly available type 2 diabetes (T2D) genome-wide association studies (GWAS) data for 70,127 subjects, we identify seven novel associated regions, five driven by common variants (LYPLAL1, NEUROG3, CAMKK2, ABO, and GIP genes), one by a low-frequency (EHMT2), and one driven by a rare variant in chromosome Xq23, rs146662075, associated with a twofold increased risk for T2D in males. rs146662075 is located within an active enhancer associated with the expression of Angiotensin II Receptor type 2 gene (AGTR2), a modulator of insulin sensitivity, and exhibits allelic specific activity in muscle cells. Beyond providing insights into the genetics and pathophysiology of T2D, these results also underscore the value of reanalyzing publicly available data using novel genetic resources and analytical approaches.
During the last decade, hundreds of genome-wide association studies (GWAS) have been performed with the aim of providing a better understanding of the biology of complex diseases, improving their risk prediction, and ultimately discovering novel therapeutic targets. However, the majority of the published GWAS have only reported primary findings, which generally explain a small fraction of the estimated heritability. To examine the missing heritability, most strategies involve generating new genetic and clinical data. Very rarely are new studies based on the revision and reanalysis of existing genetic data by applying more powerful analytic techniques and resources after the primary GWAS findings are published. These cost-effective reanalysis strategies are now possible, given emerging (1) data-sharing initiatives with large amounts of primary genetic data for multiple human genetic diseases, as well as (2) new and improved GWAS methodologies and resources. Notably, genotype imputation with novel sequence-based reference panels can now substantially increase the genetic resolution of GWASs from previously genotyped data sets, reaching good- to excellent imputation of low frequency (minor allele frequency [MAF]: 0.01 \leq \text{MAF} < 0.05) and rare variants (MAF < 0.01), increasing the power to identify novel associations, and fine map the known ones. Moreover, the availability of publicly available primary genetic data allows the homogeneous integration of multiple data sets from different origins providing more accurate meta-analysis results, particularly at the low ranges of allele frequency. Finally, the vast majority of reported GWAS analyses omits the X chromosome, despite representing 5% of the genome and coding for more than 1,500 genes. The reanalysis of publicly available data also enables interrogation of this chromosome. We hypothesized that a unified reanalysis of multiple publicly available data sets, applying homogeneous standardized quality control (QC), genotype imputation, and association methods, as well as novel and denser sequence-based reference panels for imputation would provide new insights into the genetics and the pathophysiology of complex diseases. To test this hypothesis, we focused this study on type 2 diabetes (T2D), one of the most prevalent complex diseases for which many GWAS have been performed during the past decade. These studies have allowed the identification of more than 100 independent loci, most of them driven by common variants, with a few exceptions. Despite these efforts, there is still a large fraction of genetic heritability hidden in the data, and the role of low-frequency variants, although recently proposed to be minor, has still not been fully explored. The availability of large T2D genetic data sets in combination with larger and more comprehensive genetic variation reference panels, provides the opportunity to impute a significantly increased fraction of low-frequency and rare variants, and to study their contribution to the risk of developing this disease. This strategy also allows us to fine map known associated loci, increasing the chances of finding causal variants and understanding their functional impact. We therefore gathered publicly available T2D GWAS cohorts with European ancestry, comprising a total of 13,857 T2D cases and 62,126 controls, to which we first applied harmonization and quality control protocols covering the whole genome (including the X chromosome). We then performed imputation using 1000 Genomes Project (1000G) and UK10K reference panels, followed by association testing. By using this strategy, we identified novel associated regions driven by common, low-frequency and rare variants, fine mapped and functionally annotated the existing and novel ones, allowing us to describe a regulatory mechanism disrupted by a novel rare and large-effect variant identified at the X chromosome.

Results

Overall analysis strategy. As shown in Fig. 1, we first gathered all T2D case-control GWAS individual-level data that were available through the EGA and dbGaP databases (i.e., Gene Environment-Association Studies [GENEVA], Wellcome Trust Case Control Consortium [WTCCC], Finland–United States Investigation of NIDDM Genetics [FUSION], Resource for Genetic Epidemiology Research on Aging [GERA], and Northwestern NuGENE project [NuGENE]). We harmonized these cohorts, applied standardized quality control procedures, and filtered out low-quality variants and samples (Methods and Supplementary Notes). After this process, a total of 70,127 subjects (70KforT2D, 12,931 cases, and 57,196 controls, Supplementary Data 1) were retained for downstream analysis. Each of these cohorts was then imputed to the 1000G and UK10K reference panels using an integrative method, which selected the results from the reference panel that provided the highest accuracy for each variant, according to IMPUTE2 info score (Methods). Finally, the results from each of these cohorts were meta-analyzed (Fig. 1), obtaining a total of 15,115,281 variants with good imputation quality (IMPUTE2 info score \geq 0.7, MAF \geq 0.001, and I^2 heterogeneity score \leq 0.75), across 12,931 T2D cases and 57,196 controls. Of these, 6,845,408 variants were common (MAF \geq 0.05), 3,100,848 were low-frequency (0.01 \leq \text{MAF} < 0.05), and 5,169,025 were rare (0.001 \leq \text{MAF} < 0.01). Merging the imputation results derived from the two reference panels substantially improved the number of good-quality imputed variants, particularly within the low-frequency and rare spectrum, compared to the imputation results obtained with each of the panels separately. For example, a set of 5,169,025 rare variants with good quality was obtained after integrating 1000G and UK10K results, while only 2,878,263 rare variants were imputed with 1000G and 4,066,210 with UK10K (Supplementary Fig. 1A). This strategy also allowed us to impute 1,357,753 indels with good quality (Supplementary Fig. 1B). To take full advantage of publicly available genetic data, we used three main meta-analytic approaches to adapt to the three most common strategies for genetic data sharing: individual-level genotypes, summary statistics, and single-case queries through the Type 2 Diabetes Knowledge Portal (T2D Portal) (http://www.type2diabetesgenetics.org/). We first meta-analyzed all summary statistics results from the DIAGRAM trans-ancestry meta-analysis, selecting 9,188,233 common variants (MAF \geq 0.05), mostly imputed from HapMap, with the corresponding fraction of non-overlapping samples in our 70KforT2D set, i.e. the GERA and the NuGENE cohorts, comprising a total of 7,522 cases and 50,446 controls (Fig. 1, Supplementary Data 1). Second, the remaining variants (13,197,048), which included mainly non-HapMap variants (MAF < 0.05) or variants not tested above, were meta-analyzed using all five cohorts from the 70KforT2D resource (Supplementary Data 1). Finally, low-frequency variants located in coding regions and with p \leq 1 \times 10^{-4} were meta-analyzed using the non-overlapping fraction of samples with the data from the T2D Portal through the interrogation of exome array and whole-exome sequence data from \sim 80,000 and \sim 17,000 individuals, respectively.

Pathway and functional enrichment analysis. To explore whether our results recapitulate the pathophysiology of T2D, we performed gene-set enrichment analysis with all the variants with p \leq 1 \times 10^{-5} using DEPICT (Methods). This analysis showed enrichment of genes expressed in pancreas (ranked first in tissue enrichment analysis, p = 7.8 \times 10^{-4}, FDR < 0.05, Supplementary Data 2) and cellular response to insulin stimulus (ranked second in gene-set enrichment analysis, p = 3.9 \times 10^{-5}, FDR = 0.05,
For active regulatory enhancers (Supplementary Fig.4), suggesting function, as previously reported10. Furthermore, we confirmed that the magnitude and direction of the effect of all the associated variants (p ≤ 5 × 10−8) were highly consistent with those reported previously (p = 0.92, p = 1 × 10−248. Supplementary Fig. 5). In addition, the direction of effect was consistent with all 139 previously reported variants, except three that were discovered in east and south Asian populations (Supplementary Data 4).

The high coverage of genetic variation ascertainment in this study allowed us to fine-map known and novel loci, providing more candidate causal variants for downstream functional interpretations. We constructed 99% credible variant sets11 for each of these loci, the subset of variants that have, in aggregate, 99% probability of being causal. Supplementary Data 3, Supplementary Fig. 2, Supplementary Fig. 3), in concordance with the current knowledge of the molecular basis of T2D. In addition, variant set enrichment analysis of the T2D-associated credible sets across regulatory elements defined in isolated human pancreatic islets showed a significant enrichment for active regulatory enhancers (Supplementary Fig. 4), suggesting that causal SNPs within associated regions have a regulatory function, as previously reported10.

Fine-mapping and functional characterization of T2D loci. The three association strategies allowed us to identify 57 genome-wide significant associated loci (p ≤ 5 × 10−8), of which seven were not previously reported as associated with T2D (Table 1). The remaining 50 loci have been previously reported and included, for example, two low-frequency variants recently discovered in Europeans, one located within one of the CCND2 introns (rs76895963), and a missense variant within the PAM2 gene. Furthermore, we confirmed that the magnitude and direction of the effect of all the associated variants (p ≤ 0.001) were highly consistent with those reported previously (p = 0.92, p = 1 × 10−248. Supplementary Fig. 5). In addition, the direction of effect was consistent with all 139 previously reported variants, except three that were discovered in east and south Asian populations (Supplementary Data 4).

The high coverage of genetic variation ascertainment in this study allowed us to fine-map known and novel loci, providing more candidate causal variants for downstream functional interpretations. We constructed 99% credible variant sets11 for each of these loci, i.e. the subset of variants that have, in aggregate, 99% probability of containing the true causal variant for all 57 loci (Supplementary Data 5). As an important improvement over previous T2D genetic studies, we identified small structural variants within the credible sets, consisting mostly of insertions and deletions between 1 and 1,975 nucleotides. In fact, out of the 8,348 variants included within the credible sets for these loci, 927 (11.1%) were indels, of which 105 were genome-wide significant (Supplementary Data 6). Interestingly, by integrating imputed results from 1000G and UK10K reference panels, we gained up to 41% of indels, which were only identified by either one of the two reference panels, confirming the advantage of integrating the results from both reference panels. Interestingly, 15 of the 71 previously reported loci that we replicated (p ≤ 5.3 × 10−4 after correcting for multiple testing) have an indel as the top variant, highlighting the potential role of this type of variation in the susceptibility for T2D. For example, within the IGF2BP2 intron, a well-established and functionally validated locus for T2D12, we found that 12 of the 57 variants within its 99% credible set correspond to indels with genome-wide significance (5.6 × 10−16 < p < 2.4 × 10−15), which collectively represented 18.4% posterior probability of being causal.
To prioritize causal variants within all the identified associated loci, we annotated their corresponding credible sets using the Variant Effector Predictor (VEP) for coding variants13 (Supplementary Data 8 and 9). In addition, we tested single base-pair frame-shift deletion de

Novel T2D-associated loci driven by common variants. Beyond the detailed characterization of the known T2D-associated regions, we also identified seven novel loci, among which, five were driven by common variants with modest effect sizes (1.06 < OR < 1.12; Table 1, Fig. 2, Supplementary Fig. 6 and 7).

Within the first novel T2D-associated locus in chromosome 1q41 (LYPLAL1-ZC3H11B, rs2820443-T, OR = 1.08 [1.04–1.13], p = 2.94 × 10^{-4}), several variants have been previously associated with waist-to-hip ratio, height, visceral adipose tissue in females, adiponectin levels, fasting insulin, and non-alcoholic fatty liver disease18–23. Among the genes in this locus, LYPLAL1, which encodes for lysophospholipase-like 1, appears to be the most likely effector gene, as it has been found to be downregulated in mouse models of diet-induced obesity and upregulated during adipogenesis24.

Second, a novel locus at chromosome 9q34.2 region (ABO, rs505922, OR = 1.06 [1.04–1.09], p = 4.9 × 10^{-8}) includes several variants that have been previously associated with other metabolic traits. For example, the variant rs651007, in linkage disequilibrium (LD) with rs505922 (r^2 = 0.507), has been shown to be associated with fasting glucose25, and rs146569 (r^2 with top = 1) is associated with an increased risk for cardiometabolic disorders26. One of the variants within the credible set was the single base-pair frame-shift deletion defining the blood group O27. In concordance with previous results that linked O blood type with a lower risk of developing T2D28, the frame-shift deletion determining the blood group type O was associated with a protective effect for T2D in our study (rs8176719, p = 3.4 × 10^{-4}, OR = 0.95 [0.91–0.98]). In addition, several variants within this credible set are associated with the expression of the ABO gene in multiple tissues including skeletal muscle, adipose tissue, and pancreatic islets (Supplementary Data 9, Supplementary Data 10).

A new T2D signal driven by a low-frequency variant. Furthermore, we selected all low-frequency (0.01 < MAF < 0.05) variants with p ≤ 1 × 10^{-4} in the 70KforT2D meta-analysis that

Table 1 Novel T2D-associated loci

<table>
<thead>
<tr>
<th>Novel Locus</th>
<th>Chr</th>
<th>rsID---Risk Allele</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYPLAL1/ ZC3H11B (1q41)</td>
<td>1</td>
<td>rs2820443-T</td>
<td>1.08 [1.04–1.13]</td>
<td>2.94 × 10^{-4}</td>
</tr>
<tr>
<td>EHM7</td>
<td>6</td>
<td>rs115884658-A</td>
<td>1.34 [1.18–1.53]</td>
<td>1.00 × 10^{-5}</td>
</tr>
<tr>
<td>ABO</td>
<td>9</td>
<td>rs505922-C</td>
<td>1.01 [1.03–1.11]</td>
<td>6.93 × 10^{-4}</td>
</tr>
<tr>
<td>NEUROG3 (10q22.1)</td>
<td>10</td>
<td>rs2642587-G</td>
<td>1.12 [1.08–1.16]</td>
<td>8.45 × 10^{-9}</td>
</tr>
<tr>
<td>CAMKK2 (12q24.31)</td>
<td>12</td>
<td>rs3794205-G</td>
<td>1.09 [1.05–1.14]</td>
<td>4.18 × 10^{-5}</td>
</tr>
<tr>
<td>CALCOCO2/ ATP5G1/UBE2Z/SNF5/GIP (17q12.31)</td>
<td>17</td>
<td>rs12453394-A</td>
<td>1.08 [1.04–1.12]</td>
<td>7.86 × 10^{-5}</td>
</tr>
<tr>
<td>AGTR2 (Xq23)</td>
<td>X</td>
<td>rs146662075-T</td>
<td>3.09 [2.06–4.60]</td>
<td>3.24 × 10^{-8}</td>
</tr>
</tbody>
</table>

**Notes:**

1. Chr chromosome, OR odds ratio, MAF minor allele frequency
2. Imputed based public GWAS discovery meta-analysis (NuGENE + GERA cohort, 7,522 cases and 50,446 controls)
3. Transcendancy DIAGRAM Consortium (26,688 cases and 83,964 controls)
4. Meta-P-value estimated using a weighted Z-score method due to unavailable SE information from Stage 2 replication cohorts
5. A new T2D signal driven by a low-frequency variant
6. Combined Meta-analysis bin replications
7. 70KforT2D Men Cohort (GERA cohort + GENEVA + FUSION, 5,277 cases and 15,702 controls older than 55 years)
8. Ris STRAT T2D + UK Biobank (18,370 cases and 88,283 controls older than 55 years and OGTT > 7.8 mmol l^{-1})
were annotated as altering protein sequences, according to VEP. This resulted in 15 coding variants that were meta-analyzed with exome array and whole-exome sequencing data from a total of ~97,000 individuals after excluding the overlapping cohorts between the different data sets. This analysis highlighted a novel genome-wide association driven by a low-frequency missense variant (Ser58Phe) within the network of the HLA-DQA1 locus, not only through the known rs5945326 variant (OR = 1.25, \[3.5 \times 10^{-4}\]), and in high LD with the first reported variant (\[3.5 \times 10^{-8}\]) when conditioning on the newly identified variant, rs61503151. On the other hand, when conditioning on the previously reported variant, rs5945326, the effect size of the newly identified indel remained significant and with a larger effect size (OR = 1.33, \(p = 0.003\)), placing this deletion, as a more likely candidate causal variant for this locus (Supplementary Data 14).

In addition, we identified a novel genome-wide significant signal in males at the Xq23 locus driven by a rare variant (rs146662075, MAF = 0.008, OR = 2.94 \[2.00–4.31\], \(p = 3.5 \times 10^{-5}\); Fig. 3a). Two other variants in LD with the top variant, rs139246371 (chrX:115329804, OR = 1.65, \(p = 3.5 \times 10^{-5}\), \(r^2 =\) 0.62) showed that the originally reported variant was no longer significant (OR = 1.01, \(p = 0.94\)) when conditioning on the newly identified variant, rs61503151. The other hand, when conditioning on the previously reported variant, rs5945326, the effect of the newly identified indel remained significant and with a larger effect size (OR = 1.33, \(p = 0.003\)), placing this deletion, as a more likely candidate causal variant for this locus (Supplementary Data 14).

A novel rare X chromosome variant associated with T2D. Similar to other complex diseases, the majority of published large-scale T2D GWAS studies have omitted the analysis of the X chromosome, with the notable exception of the identification of a T2D-associated region near the DUSP9 gene in 2010. To fill this gap, we tested the X chromosome genetic variation for association with T2D. To account for heterogeneity of the effects and for the differences in imputation performance between males and females, the association was stratified by sex and tested separately, and then meta-analyzed. This analysis was able to replicate the DUSP9 locus, not only through the known rs5945326 variant (OR = 1.15, \(p = 0.049\)), but also through a three-nucleotide deletion located within a region with several promoter marks in liver (rs61503151 [GCCA/G], OR = 1.25, \(p = 3.5 \times 10^{-4}\)), and in high LD with the first reported variant (\(r^2 = 0.62\)). Conditional analyses showed that the originally reported variant was no longer significant (OR = 1.01, \(p = 0.94\)) when conditioning on the newly identified variant, rs61503151. On the other hand, when conditioning on the previously reported variant, rs5945326, the effect size of the newly identified indel remained significant and with a larger effect size (OR = 1.33, \(p = 0.003\)), placing this deletion, as a more likely candidate causal variant for this locus (Supplementary Data 14).

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0.37 with the top variant) and rs6603744 (chrX:115823966, \( OR = 1.28, p = 1.7 \times 10^{-4}, r^2 = 0.1 \) with the top variant), comprised the 99% credible set and supported the association. We tested in detail the accuracy of the imputation for the rs146662075 variant by comparing the imputed results from the same individuals genotyped by two different platforms (Methods) and found that the imputation was highly accurate in males only when using UK10K, but not in females, nor when using 1000G \( (R^2_{[UK10K,males]} = 0.94, R^2_{[UK10K,females]} = 0.66, R^2_{[1000G,males]} = 0.62, \) and \( R^2_{[1000G,females]} = 0.43; \) Supplementary Fig. 8). Whether this association is specific to men, or whether it also affects female carriers, remains to be clarified with datasets that allow accurate imputation on females, or with direct genotyping or sequencing.

To further validate and replicate this association, we next analyzed four independent data sets (SIGMA\(^6\), INTERACT\(^{43}\), Partners Biobank\(^{44}\), and UK Biobank\(^{45}\)), by performing imputation with the UK10K reference panel. In addition, a fifth cohort was genotyped de novo for the rs146662075 variant in several Danish sample sets. The initial meta-analysis, including the five replication data sets did not reach genome-wide significance \( (OR = 1.57, p = 1.2 \times 10^{-5}; \) Supplementary Fig. 9A), and revealed a strong degree of heterogeneity (heterogeneity \( \chi^2 = 0.004 \)), which appeared to be driven by the replication cohorts.

As a complementary replication analysis, within one of the case-control studies, there was a nested prospective cohort study, the Inter99, which consisted of 1,652 nondiabetic male subjects genotyped for rs146662075, of which 158 developed T2D after 11 years of follow-up. Analysis of incident diabetes in this cohort confirmed the association with the same allele, as previously seen in the case-control studies, with carriers of the rare T allele having increased risk of developing incident diabetes, compared to the C carriers (Cox-proportional hazards ratio (HR) = 3.17 [1.3–7.7], \( p = 0.0111 \), Fig. 3b). Nearly 30% of carriers of the T risk allele developed incident T2D during 11 years of follow-up, compared to only 10% of noncarriers.

To understand the strong degree of heterogeneity observed after adding the replication datasets, we compared the clinical and demographic characteristics of the discovery and replication cohorts, and found that the majority of the replication datasets contained control subjects that were significantly younger than 55 years, the average age at the onset of T2D reported in this study and in Caucasian populations\(^{46}\). This was particularly clear for the Danish cohort \( (age \text{ cases} [95\%CI] = 46.9 [46.6–47.2] \text{ vs. age \text{ cases} [95\%CI] = 60.7 [60.4–61.0])} \) and for INTERACT \( (age \text{ controls} [95\%CI] = 51.7 [51.4–52.1] \text{ vs. age \text{ cases} [95\%CI] = 54.8 [54.6–55.1]; Supplementary Fig. 10)\). Given the supporting results from the Inter99 prospective cohort, we performed an additional analysis using a stricter definition of controls, to minimize the presence of prediabetics or individuals that may further develop diabetes after reaching the average age at the onset. For this, we applied two additional exclusion criteria: (i) subjects younger than 55 years and (ii), when possible, excluding individuals with measured 2-h plasma glucose values during oral glucose tolerance test (OGTT) above 7.8 mmol\( L^{-1}\), a threshold employed to identify impaired glucose tolerance (prediabetes)\(^{47}\), or controls with family history of T2D, both being strong risk factors for developing T2D. While the application of the first filter alone did not yield genome-wide significant results (Supplementary Fig. 9B), upon excluding individuals with prediabetes or a family history of T2D, the replication results were significant and consistent with the initial discovery results \( (OR = 1.57 [1.19–2.07], p = 0.0014) \). The combined analysis of the discovery and replication cohorts resulted in genome-wide significance, confirming the association of rs146662075 with T2D \( (OR = 1.95 [1.56–2.45], p = 7.8 \times 10^{-5}; \) Fig. 3c).

Allele-specific enhancer activity of the rs146662075 variant. We next explored the possible molecular mechanism behind this association, by using different genomic resources and experimental approaches. The credible set of this region contained three variants, with the leading SNP alone (rs146662075), showing 78% posterior probability of being causal \( (Supplementary \text{ Fig. 7, Supplementary Data 5})\), as well as the highest CADD (scaled C-score = 15.68; Supplementary Data 8), and LINSIGHT score \( (Supplementary \text{ Data 9})\). rs146662075 lies within a chromosomal region enriched in regulatory (DNase I) and active enhancer (H3K27ac) marks, between the AGTR2 \( (at 103 \text{ kb})\) and the SLC6A14 \( (at 150 \text{ kb})\) genes. The closest gene AGTR2, which encodes for the angiotensin II receptor type 2, has been previously associated with insulin secretion and resistance\(^{48–50}\). From the analysis of available epigenomic data sets\(^{51}\), we found no evidences of H3K27ac or other enhancer regulatory marks in human pancreatic islets; whereas a significant association was observed between the presence of H3K27ac enhancer marks and the expression of AGTR2 across multiple tissues \( (Fisher \text{ test} p = 4.45 \times 10^{-3})\). Among T2D cases, we tested in detail the accuracy of the imputation for the rs146662075 variant could act as a transcriptional enhancer and whether its activity was allele-specific. For this, we linked the DNA region with either the T (risk) or the C (non-risk) allele, to a minimal promoter and performed luciferase assays in a mouse myoblast cell line. The luciferase analysis showed an average 4.4-fold increased activity for the disease-associated T allele, compared to the expression measured with the common C allele, suggesting an activating function of the T allele, or a repressive function of the C allele \( (Fig. 4b)\). Consistent with these findings, electrophoretic mobility shift assays using nuclear protein extracts from mouse myoblast cell lines, differentiated myotubes, and human fetal muscle cell line, revealed sequence-specific binding activity of the C allele, but not the rare T allele \( (Fig. 4c)\).

We next studied whether the region encompassing the rs146662075 variant could act as a transcriptional enhancer and whether its activity was allele-specific. For this, we linked the DNA region with either the T (risk) or the C (non-risk) allele, to a minimal promoter and performed luciferase assays in a mouse myoblast cell line. The luciferase analysis showed an average 4.4-fold increased activity for the disease-associated T allele, compared to the expression measured with the common C allele, suggesting an activating function of the T allele, or a repressive function of the C allele \( (Fig. 4b)\). Consistent with these findings, electrophoretic mobility shift assays using nuclear protein extracts from mouse myoblast cell lines, differentiated myotubes, and human fetal muscle cell line, revealed sequence-specific binding activity of the C allele, but not the rare T allele \( (Fig. 4c)\).

Overall, these data indicate that the risk T allele prevents the binding of a nuclear protein that is associated with decreased activity of an AGTR2-linked enhancer.

Discussion

Through harmonizing and reanalyzing publicly available T2D GWAS data, and performing genotype imputation with two whole-genome sequence-based reference panels, we are able to perform deeper exploration of the genetic architecture of T2D. This strategy allowed us to impute and test for association with T2D more than 15 million of high-quality imputed variants, including low-frequency, rare, and small insertions and deletions, across chromosomes 1–22 and X.

The reanalysis of these data confirmed a large fraction of already-known T2D loci, and identified novel potential causal variants by fine mapping and functionally annotating each locus.

This reanalysis also allowed us to identify seven novel associations, five driven by common variants in or near \( LYPPL1, NEUROG3, CAMKK2, ABO, \) and \( GIP; \) a low-frequency variant in \( EHM7T2, \) and a rare variant in the X chromosome. This rare variant identified in \( Xq23 \) chromosome was located near the AGTR2 gene, and showed nearly twofold increased risk for T2D in males, which represents, to our knowledge, the largest effect size identified so far in Europeans, and a magnitude similar to other variants with large effects identified in other populations\(^{52,53}\).

Our study complemented other efforts that also aim at unraveling the genetics behind T2D through the generation of new
**Fig. 3** Discovery and replication of rs14666075 association signal. 

**a** Forest plot of the discovery of rs146662075 variant. Cohort-specific odds ratios are denoted by boxes proportional to the size of the cohort and 95% CI error bars. The combined OR estimated for all the data sets is represented by a diamond, where the diamond width corresponds to 95% CI bounds. The p-value for the meta-analysis (Meta P) and for the heterogeneity (Het P) of odds ratio is shown. 

**b** Kaplan–Meier plot showing the cumulative incidence of T2D for a 11 years follow-up. The red line represents the T carriers and in light blue, C carriers are represented (n = 1,652, cases = 158). 

**c** Forest plot after excluding controls younger than 55 years, OGTT > 7.8 mmol l\(^{-1}\), and controls with family history of T2D in both the discovery and replication cohorts when available.
genetic data. For example, we provided for the first time a comprehensive coverage of structural variants, which point to previously unobserved candidate causal variants in known and novel loci, as well as a comprehensive coverage of the X chromosome through sequence-based imputation.

This study also highlights the importance of a strict classification of both cases and controls, in order to identify rare variants associated with disease. Our initial discovery of the Xq23 locus was only replicated when the control group was restricted to T2D-free individuals who were older than 55 years (average age...
at the onset of T2D), had normal glucose tolerance, and no family history of T2D. This is in line with previous results obtained for a T2D population-specific variant found in Inuit within the TBC1D4 gene, which was only significant when using OGTT as criteria for classifying cases and controls, but not when using HbA1c. Our observation that 30% of the rs146662075-C risk allele carriers developed T2D over 11 years of follow-up, compared to 10% of noncarriers, further supports the association of this variant and suggests that an early identification of these subjects through genotyping may be useful to tailor pharmacological or lifestyle intervention to prevent or delay the onset of T2D.

Using binding and gene-reporter analyses, we demonstrated a functional role of this variant and proposed a possible mechanism behind the pathophysiology of T2D in T risk allele carriers, in which this rare variant could favor a gain of function of AGTR2, previously associated with insulin resistance. AGTR2 appears, therefore, as a potential therapeutic target for this disease, which would be in line with previous studies showing that the blockade of the renin-angiotensin system in mice and in humans prevents the onset of T2D, and may favor weight loss.

Overall, beyond our significant contribution toward expanding the number of genetic associations with T2D, our study also highlights the potential of the reanalysis of public data, as a complement to large studies that use newly generated data. This study informs the open debate in favor of data sharing and democratization initiatives, for investigating the genetics and pathophysiology of complex diseases, which may lead to new preventive and therapeutic applications.

Methods

Quality filtering for imputed variants. In order to assess genotype imputation quality and to determine an accurate post-imputation quality filter, we made use of the Wellcome Trust Case Control Consortium (WTCCC) data available through the European Genotype Archive (EGA, https://www.ebi.ac.uk/ega/studies/EGAS000000000028). The genotyping data and the subjects included in the following tests were filtered according to the guidelines provided by the WTCCC, whose criteria of exclusion are in line with standard quality filters for GWAS. We used the 1958 British Birth cohort (~3,000 samples, 58C) that was genotyped by Affymetrix v6.0 and Illumina i12M chips. After applying the quality filtering criteria, 2,706 and 2,699 subjects from the Affymetrix and Illumina data, respectively, were available for the 58C samples, leaving an intersection of 2,509 individuals genotyped by both platforms. After variant quality filtering and excluding all the variants with minor allele frequency (MAF) below 0.01, 717,556, and 892,516 variants remained for 58C Affymetrix and Illumina platforms, respectively.

We used a two-step genotype imputation approach based on prephasing the study genotypes into full haplotypes with SHAPEIT2 to ameliorate the computational burden required for genotype imputation through IMPUTE2. We used the GTOOL software (http://www.well.ox.ac.uk/~cfreeman/software/gwas/gtool.html, version 0.7.5) to homogenize strand annotation by merging the imputed results obtained from each set of genotyped data. To ensure that there were no strand orientation issues, we excluded all C/G and A/T SNPs. To perform genotype imputation, we used two sequence-based reference panels: the 1000G and UK10K reference panels by choosing for each variant, the reference panel that provided the best IMPUTE2 info score. For 1000G-based genotype imputation in chromosome X (chrX), we used the ‘V3, macGT1’ release (August, 2012). For chrX, we restricted the analysis to non-pseudoautosomal (non-PAR) regions and stratified the association analysis by sex to account for hemizygosity for males, while for females, we followed an autosomal model. Also, we did not apply HWE filtering in the X chromosome variants.

Finally, for the GERA cohort due to the large computational burden that comprises the whole genotype imputation process in such a large sample size, we randomly split this cohort into two homogeneous subsets of ~30,000 individuals each, in order to minimize the memory requirements.

We included variants with IMPUTE2 INFO score ≥ 0.7, MAF ≥ 0.01%, and for autosomal variants, HWE controls p > 1 × 10^{-6}. Further details about genotype imputation and covariate information used in association testing are summarized in Supplementary Data 1.

70KforT2D and inclusion of previous summary statistics data. We meta-analyzed the different sets from the 70KforT2D data set with METAL, using the inverse variance-weighted fixed effect model. We included variants with F heterozygosity < 0.75. This filter was not applied to the final X chromosome data set, after meta-analyzing the results from males and females separately (which were already filtered by F < 0.75).

For the meta-analysis with the DIAGRAM trans-ethnic study, we excluded from the whole 70KforT2D datasets those cohorts that overlapped with the DIAGRAM data. Therefore, we meta-analyzed the GERA and NuGENE cohorts (7,522 cases and 50,446 controls) and the 70KforT2D resource (58C individuals that were genotyped by both independent genotyping platforms). For SNP genotyping, we used the GTOOL software (http://www.well.ox.ac.uk/~cfreeman/software/gwas/gtool.html, version 0.7.5). For the meta-analysis, we used two sequence-based reference panels: the 1000G and UK10K reference panels by choosing for each variant, the reference panel that provided the best IMPUTE2 info score. For 1000G-based genotype imputation in chromosome X (chrX), we used the ‘V3, macGT1’ release (August, 2012). For chrX, we restricted the analysis to non-pseudoautosomal (non-PAR) regions and stratified the association analysis by sex to account for hemizygosity for males, while for females, we followed an autosomal model. Also, we did not apply HWE filtering in the X chromosome variants.

Finally, for the GERA cohort due to the large computational burden that comprises the whole genotype imputation process in such a large sample size, we randomly split this cohort into two homogeneous subsets of ~30,000 individuals each, in order to minimize the memory requirements.

We included variants with IMPUTE2 INFO score ≥ 0.7, MAF ≥ 0.01%, and for autosomal variants, HWE controls p > 1 × 10^{-6}. Further details about genotype imputation and covariate information used in association testing are summarized in Supplementary Data 1.
DIAGRAM trans-ethnic meta-analysis, we performed a sample size based meta-analysis, which converts the direction of the effect and the p-value into a Z-score. In addition, we also performed an inverse variance-weighted fixed effect meta-analysis to estimate the final effect sizes. This approach required the estimation of the beta and standard errors from the summary statistics (p-value and odds ratio). For the meta-analysis of coding low-frequency variants with the Type 2 Diabetes Knowledge Portal (T2D Portal), we included the 70% of the data set the NuGENE and GERA cohorts (7,522 cases and 50,446 controls), to avoid overlapping samples. Like in the previous scenario, standard errors were not provided for the T2D Portal data and we used a sample size based meta-analysis with METAL. However, to estimate the effect sizes, we also calculated the standard errors from the p-values and odds ratios, and we performed an inverse variance-weighted fixed effect meta-analysis.

See further details about the cohorts in Supplementary Note 1.

Pathway and enrichment analysis. Summary statistics that resulted from the 70% of the meta-analysis were analyzed by Data-driven Expression-Prioritized Integration for Complex Traits (DEPICT)10 to prioritize likely causal genes, to highlight enriched pathways, and to identify the most relevant tissues/cell types. DEPICT relies on publicly available gene sets (including molecular pathways) and leverages gene expression data from 78,840 gene expression arrays, to perform gene prioritization and gene-set enrichment based on predicted gene function and the so-called reconstituted gene sets. A reconstituted gene set contains a membership probability for each gene and conversely, each gene is functionally characterized by its membership in one or more gene sets. False discovery rate (FDR) was estimated using 10,000 permutations. Significance was calculated using the Fisher exact test. We used all summary statistics from autosomal variants with p < 1 × 10⁻⁵ in the 70% of the meta-analysis. We used an updated version of DEPICT, which handled 100,000 Phase1-integrated haplotypes (June 2014, www.broadinstitute.org/depict). DEPICT was run using 3,412 associated SNPs (p < 1 × 10⁻⁵), from which we identified independent SNPs using PLINK and the following parameters: --clump-p1 5e-8, --clump-p2 1e-5, --clump-r2 0.6, and --clump-kb 250. We used LD r² > 0.5 distance to define locus limits yielding 70 autosomal loci comprising 119 genes (note that this is not the same locus definition used in the literature). We ran DEPICT with default settings, i.e., using 500 permutations to adjust for bias and 50 replications to estimate false discovery rate (FDR). We used normalized expression data from 78,840 Affymetrix microarrays to reconstitute gene sets9. The resulting 14,461 reconstituted gene sets were tested for enrichment analysis. A total of 209 tissue or cell type expression data sets assembled from 37,427 Affymetrix U133 Plus 2.0 Array samples were used for enrichment in tissue/cell-type expression data sets. DEPICT identified 103 reconstituted gene sets significantly enriched (FDR < 0.05) for genes found among the 70 loci associated to T2D. We did not reconstitute reconstituted sets in which genes of the original gene set were not nominally enriched (Wilcoxon rank-sum test), as these are expected to be enriched in the reconstituted gene set by design. The lack of enrichment makes the interpretation of the reconstituted gene set challenging because the label of the reconstituted gene set will not be accurate. Hence, the following reconstituted gene sets were removed from the results (Wilcoxon rank sum and p-values in parentheses): MP:00024247 gene set (p = 0.73), GO:0070491 gene set (p = 0.14), MP:00020165 gene set (p = 0.17), MP:0005491 gene set (p = 0.54), GO:0005159 gene set (p = 0.06), MP:00001269 gene set (p = 0.03), MP:0006344 gene set (p = 0.42), MP:0004188 gene set (p = 0.22), MP:0002189 gene set (p = 0.02), MP:0000003 gene set (p = 0.08), ENSG00000166604 gene set (p = 0.13), GO:0005158 gene set (p = 0.07), and MP:0001715 gene set (p = 0.01). After applying the filters described above, there were 89 significantly enriched reconstituted gene sets. We used the affinity projection tool to cluster related reconstituted gene sets (network diagram script available from https://github.com/perslab/DEPICT).

We also used the VSE R package to compute the enrichment or depletion of genetic variants comprised in the 57 credible sets listed in Supplementary Data5. We examined whether indels from the 99% credible sets were present or absent in the 1000G Phase1 or UK10K reference panels, and also checked whether they were present or not in the 1000G Phase3 reference panel. All the information has been summarized in Supplementary Data5. We also visually inspected the aligned reads of the most variant indels from both projects to discard that they could be alignment artifacts.

Functional annotation of the 99% credible sets variants. To determine the effect of 99% credible sets variants on genes, transcripts, and protein sequence, we used the variant effect predictor (VEP, GRCh37.p13 assembly)11. The VEP application determines the effect of variants (SNPs, insertions, deletions, CNVs, or structural variants) on genes, transcripts, proteins, and regulatory regions. We used as input the coordinates of variants within 99% credible sets and the corresponding alleles, to find out the affected genes and RefSeq transcripts and the consequence on the protein sequence by using the GRCh37.p13 assembly. We also manually checked all these annotations with the Exome Aggregation Consortium data set (ExAC, http://exac.broadinstitute.org) and the most updated VEP server based on the GRCh38.p7 assembly. All these annotations are provided in Supplementary Data7.

We used combined annotation-dependent depletion (CADD) scoring function to prioritize functional, deleterious, and disease causal variants. We obtained the scaled C-score (PHRED-like scaled C-score ranking each variant with respect to all possible substitutions of the human genome) metric for each 99% credible set variant, as it highly ranks causal variants within individual genome sequences12 (Supplementary Data8). We also used the LINSIGHT score to prioritize functional variants, which measures the probability of negative selection on noncoding sites by combining a generalized linear model for functional genomic data with a probabilistic model of molecular evolution13. For each credible set variant, we retrieved the precomputed LINSIGHT score at that particular nucleotide site, as well as the mean LINSIGHT precomputed score for a region of 20 bp centered on each credible set variant, respectively (https://github.com/CdbiSiepelLab/LINSIGHT). These metrics are summarized in Supplementary Data9.

In order to prioritize functional regulatory variants, we used the V6 release from the GTEX data that provides gene-level expression quantifications and eQTL results based on the annotation with GENCODE v19. This release included 450 genotyped donors, 8,555 RNA-seq samples across 51 tissues, and two cell lines, which led to the identification of eQTLs across 44 tissues14. Moreover, RNA-seq data from human pancreatic islets from 89 deceased donors cataloged as eQTLs and exome use (sQTL) were also integrated with the GWAS data to prioritize candidate regulatory variants15 but in pancreatic islets, which is a target tissue for T2D. Both analyses are summarized in Supplementary Data10 and Supplementary Data11, respectively.

Conditional analysis. To confirm the independence between novel loci and previously known T2D signals, we performed reciprocal conditional analyses (Supplementary Data5, Supplementary Data12, Supplementary Data13, and Supplementary Data14). We included the conditioning SNP as a covariate in the causal SNP if this has been genotyped or imputed. The credible set construction provides, for each variant placed within a certain associated locus, a posterior probability of being the causal one11. We estimated the approximate Bayes factor (ABF) for each variant as

\[
\text{ABF} = \sqrt{1 - p_{\text{multi}}}^{1/2},
\]

where

\[
0.04 < r = \frac{\text{SE} + 0.04}{\text{SE}},
\]

and the SE are the estimated effect size and the corresponding standard error resulting from testing for association under a logistic regression model. The posterior probability for each variant was obtained as

\[
\text{Posterior Probability}_{i} = \frac{\text{ABF}_{i}}{\text{ABF} + 1},
\]

where ABF corresponds to the approximate Bayes factor for the marker i and T represents the sum of all the ABF values from the candidate variants enclosed in the interval being evaluated. This calculation assumes that the prior of the \( \beta \) corresponds to a Gaussian with mean 0 and variance 0.04, which is also the prior commonly employed by SNPTEST, the program being used for calculating association with eQTLs.

Finally, we ranked variants according to the ABF (in decreasing order) and from this ordered list, we calculated the cumulative posterior probability. We included variants in the 99% credible set of each region until the SNP that pushed the cumulative posterior probability of association over 0.99.

The 99% credible sets of variants for each of the 57 GWAS-significant regions are summarized in Supplementary Data5.

Characteristic of indels. We examined whether indels from the 99% credible sets were present or absent in the 1000G Phase1 or UK10K reference panels, and also checked whether they were present or not in the 1000G Phase3 reference panel. All the information has been summarized in Supplementary Data5. We also visually inspected the aligned reads of the most variant indels from both projects to discard that they could be alignment artifacts.
logistic regression model, assuming that every residual signal that arises corresponds to a secondary signal independent from this conditioning SNP. We applied this methodology to the HLA DRB1 locus (less than 1MB away from the region where T2D and T1D signals have been identified), to confirm that this association was independent of previously reported T2D signals and also to discard that this association is also driven by possible contamination of T1D diagnosed as T2D cases. We conditioned on the variant identified in this study and the top variant from the 99% credible set analysis, but also on the top variant previously described for T2D and T1D. For this purpose, we used the full 70KforT2D resource (NuGENE, GERA, FUSION, GENEVA, and WTCCC cohorts imputed with 1000G and UK10K reference panels). Finally, all the results were meta-analyzed as explained in previous sections. These analyses are provided in Supplementary Data 13, which allowed us to build 99% credible sets based on the results from the conditional analyses (included in Supplementary Data 5), and allowed us to identify the most likely causal variant for the DUSP9 locus (Supplementary Data 14).

Replication of the rare variant association at Xq23. To replicate the association of the rs146662075 variant, we performed genotype imputation with the UK10K reference panel in four independent data sets: the IntAct case-cohort study41, the Slim Initiative in Genomic Medicine for the Americas (SIGMA) consortium GWAS66, the Danish National Health Care Cohorts (Partners Biobank) data set74, and the UK Biobank cohort45. Phasing was performed with SHAPEIT2 and the IMPUTE2 software was used for genotype imputation.

The current UK Biobank data release did not contain imputed data for the X chromosome, for which phasing and imputation had to be analyzed in-house. The data were used to test the X chromosome genotypes of 488,377 participants, which were assayed using two arrays sharing 95% of marker content (Applied Biosystems™ UK BiLeVE Axiom™ Array and the Applied Biosystems™ UK Biobank Axiom™ Array). We included samples and markers that were used as input for phasing by UK Biobank investigators. At the sample level, we also excluded individuals with missing call rate > 10% or phasing discordance between the reported and the genetically predicted sex. At the variant level, we excluded markers with MAF < 0.1% and with missing call rate > 5%. The final set of 16,463 X chromosome markers and 222,725 male individuals was split into six subsets due to the huge computational burden that would require phasing the entire data set. We excluded the data set excluded marker variants with MAF < 1%, and variants showing deviation of Hardy–Weinberg equilibrium with p < 1 x 10−26 before the imputation step. In addition, from those pairs of relatives reported to be third degree or higher according to UK Biobank, we excluded from each pair the individual with the lowest call rate. We then tested the rs146662075 variant for association with type 2 diabetes using SNPTEST v2.5.1 and Fisher’s exact test.

Allele-specific enhancer activity at rs146662075. The mouse C2C12 cell line (ATCC CRL-1772) was grown in DMEM medium supplemented with 10% FBS and was induced to differentiate in DMEM with 10% horse serum for 4 days. The human myoblast myoblast cell line was established by Prof. Giulio Cossu (Institute of Inflammation and Repair, University of Manchester). The authors played no role in the procurement of the tissue. Cells were cultured in DMEM medium supplemented with 10% fetal calf serum and was induced to differentiate in DMEM with 2% horse serum for 4 days.

To perform an electrophoretic mobility shift assay, nuclear extracts from mouse myoblasts (C2C12 cell line) and the human myoblast cell line (ATCC CRL-1772) were obtained as described elsewhere. Double-stranded oligonucleotides containing either the common or rare variants of rs146662075 were labeled using dCTP [α-32P] (Perkin Elmer). Oligonucleotide sequences are as follows (SNP location is underlined): probe-C: 5′-gactTtGgagaACcCaGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgGgG

Acknowledgements
This work has been sponsored by the grant SEV-2011-00067 of Severo Ochoa Program, awarded by the Spanish Government. This work was supported by an EFSD/Lilly research fellowship. Josep M. Mercader was supported by Sara Berrrell Fellowship from the Instituto Carlos III and Beatrúpi de Pinós fellowship from the Agency for Management of University and Research Grants (AGAUR). Silvia Bonàs was FI DGR Fellowship from FEs DGR 2013 from Agència de Gestió d’Ajusts Universitaris i de Recerca (AGAUR, Generalitat de Catalunya). This study makes use of data generated by the WTCCC. A full list of the investigators who contributed to the generation of the data is available from www.wtccc.org.uk. Funding for the project was provided by the Wellcome Trust under award 076113. This study also makes use of data generated by the UK10K Consortium, derived from samples from UK10K COHORT IMITATION (EAS00001000713). A full list of the investigators who contributed to the generation of the data is available from www.uk10k.org. Funding for UK10K was provided by the Wellcome Trust under award WT091310. We acknowledge PRACE for awarding us to access MareNostrum supercomputer, based in Spain at Barcelona. The technical support group, particularly Pablo Rödenas and Jorge Rodríguez, from the Barcelona Supercomputing Center is gratefully acknowledged. This project has received funding from the European Union’s Horizon 2020 research and innovation program under grant agreement No 667191. Merce Planas-Félix is funded by the Obra Social Fundació la Caixa fellowship under the Severo Ochoa 2013 program. Work from Irene Miguel-Escalada, Ignasi Moran, Goutham Atlal, and Jorge Ferrer was supported by the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre, the Wellcome Trust (WT101033), Ministerio de Economía y Competitividad (BFU2014-54284-R) and Horizon 2020 (667191). We acknowledge Prof. Giulio Cossu (Institute of Inflammation and Repair, University of Manchester) for providing the muscle myoblast cell line. We also acknowledge the InterAct and SIGMA Type 2 Diabetes Consortia for access to the data to replicate the rs146662075 variant. A full list of the investigators of the SIGMA Type 2 Diabetes and the InterAct consortia is provided in Supplementary Notes 3 and 4. The Novo Nordisk Foundation Center for Basic Metabolic Research is an independent research center at the University of Copenhagen partially funded by an unrestricted donation from the Novo Nordisk Foundation (www.metabol.ku.dk). This has been conducted using the UK Biobank Resource (application number 16803). We also acknowledge Bianca C. Porneala, MS for his technical assistance in the collection and curation of the genotype and phenotype data from Partners Biobank. We also thank Marcín von Grothuss for their support for uploading the summary statistics data to the Type 2 Diabetes Genetic Portal (AMP-T2D portal). Finally, we thank all the Computational Genomics group at the BSC for their helpful discussions and valuable comments on the manuscript.

Author contributions

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-017-02380-9.

Competing interests: The authors declare no competing financial interests.

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