Long-range DNA looping and gene expression analyses identify DEXI as an autoimmune disease candidate gene

Lucy J. Davison¹,*, Chris Wallace¹, Jason D. Cooper¹, Nathan F. Cope³, Nicola K. Wilson², Deborah J. Smyth¹, Joanna M.M. Howson¹, Nada Saleh¹, Abdullah Al-Jeffery¹, Karen L. Angus¹, Helen E. Stevens¹, Sarah Nuttland¹, Simon Duley¹, Richard M.R. Coulson¹, Neil M. Walker¹, Oliver S. Burren¹, Catherine M. Rice⁴, Francois Cambien⁵, Tanja Zeller⁶, Thomas Munzel⁷, Karl Lackner⁸, Stefan Blankenberg⁶, The Cardiogenics Consortium†, Peter Fraser³, Berthold Gottgens² and John A. Todd¹

¹Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Department of Medical Genetics and ²Haematopoetic Stem Cell Lab, Cambridge Institute for Medical Research (CIMR), NIHR Biomedical Research Centre, University of Cambridge, Cambridge, UK, ³Nuclear Dynamics Laboratory, Babraham Institute, Cambridge, UK, ⁴The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK, ⁵INSEMr UMR 937, Pierre and Marie Curie University and Medical School, Paris, France, ⁶University Heart Center Hamburg, Clinical for General and Interventional Cardiology, 20246 Hamburg, Germany, ⁷Medizinische Klinik und Poliklinik, Johannes-Gutenberg Universität Mainz, Germany and ⁸Department of Clinical Chemistry and Laboratory Medicine, Johannes-Gutenberg Universität Mainz, Germany

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The chromosome 16p13 region has been associated with several autoimmune diseases, including type 1 diabetes (T1D) and multiple sclerosis (MS). CLEC16A has been reported as the most likely candidate gene in the region, since it contains the most disease-associated single-nucleotide polymorphisms (SNPs), as well as an immunoreceptor tyrosine-based activation motif. However, here we report that intron 19 of CLEC16A, containing the most autoimmune disease-associated SNPs, appears to behave as a regulatory sequence, affecting the expression of a neighbouring gene, DEXI. The CLEC16A alleles that are protective from T1D and MS are associated with increased expression of DEXI, and no other genes in the region, in two independent monocyte gene expression data sets. Critically, using chromosome conformation capture (3C), we identified physical proximity between the DEXI promoter region and intron 19 of CLEC16A, separated by a loop of >150 kb. In reciprocal experiments, a 20 kb fragment of intron 19 of CLEC16A, containing SNPs associated with T1D and MS, as well as with DEXI expression, interacted with the promoter region of DEXI but not with candidate DNA fragments containing other potential causal genes in the region, including CLEC16A. Intron 19 of CLEC16A is highly enriched for transcription-factor-binding events and markers associated with enhancer activity. Taken together, these data indicate that although the causal variants in the 16p13 region lie within CLEC16A, DEXI is an unappreciated autoimmune disease candidate gene, and illustrate the power of the 3C approach in progressing from genome-wide association studies results to candidate causal genes.

*To whom correspondence should be addressed at: JDRF/WT Diabetes and Inflammation Laboratory, Department of Medical Genetics, Cambridge Institute for Medical Research, Hills Road, Cambridge CB2 0XY, UK. Tel: +44 1223762104; Fax: +44 1223762102; Email: lucy.davison@cimr.cam.ac.uk
†Full list of Cardiogenics contributors is given in Supplementary Material.

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INTRODUCTION

The chromosome 16p13 region of the human genome has gained increasing attention since it was first associated with risk of type 1 diabetes (T1D) by genome-wide association study (GWAS) and fine mapping in 2007 (1–3). Subsequent association and candidate gene studies, in other autoimmune diseases such as multiple sclerosis (MS) (4–6), Addison’s disease (7), primary biliary cirrhosis (8) and systemic lupus erythematosus (SLE) (9,10), have also demonstrated association of this region with disease risk, implying that the 16p13 region contains a key regulator of the self-reactive immune response.

The 16p13 region is dominated by the large, 238 kb CLEC16A gene (previously known as KIAA0330). The most highly disease-associated single-nucleotide polymorphisms (SNPs) lie predominantly within the 60 kb intron 19 of CLEC16A (1,2,4,5,10,11), as well as within intron 10 of the same gene. The most commonly cited disease-associated SNPs within these respective introns are rs12708716 and rs8062322, which are in high linkage disequilibrium (LD) and are, therefore, likely to be tagging the same signal. There is also evidence of a second T1D association signal in the 16p13 region (3,12), 3’ of CLEC16A, within the C16orf75 gene. This second disease-association signal was detected in the absence of the intron 19/intron 10 signal in celiac disease (13,14), and is likely to be related to an independent causal variant common to both celiac disease and T1D. The complexity of the region is also highlighted by a recent report that the 16p13 region harbours three independent MS-associated loci (6).

CLEC16A is regarded as a potential causal gene, since it contains an immunoreceptor tyrosine-based activation motif (ITAM) (3) and is widely expressed in cells of the immune system; we note that the C-lectin-binding function implied by its name remains questionable since it is only 22 amino acids long. A recent study suggested that MS-associated SNPs in CLEC16A were correlated with relative expression of two CLEC16A isoforms in thymus but not in peripheral blood (15). However, there are several genes in the 16p13 region, in addition to CLEC16A. These include some attractive candidate genes for T1D and/or MS risk, such as MHC class II transactivator (CIITA) and suppressor of cytokine signalling 1 (SOCS1) (Fig. 1), as well as DEXI, a gene of unknown function, which has not previously been regarded as a strong autoimmune candidate gene.

Identification of the causal variant(s) and gene(s) presents a particular challenge when there is a high degree of LD in a disease-associated region and also when there are multiple genes in a region, some of which have an unknown function. If the expression of a particular gene is correlated with disease-associated SNPs, this strengthens the candidacy of that gene for an active role in the pathogenesis of disease. We used published and unpublished human monocyte gene expression data to examine expression quantitative trait loci (eQTLs) within the 16p13 region of autoimmune disease association. Interrogation of the recently published ChIP-Seq ENCODE data sets was undertaken to identify enhancer, transcription-factor-binding and RNA-polymerase-II-binding marks in the 16p13.13 region, which were consistent with the role of intron 19 of CLEC16A as a regulatory sequence.

Gene expression analysis was followed by chromosome conformation capture (3C) experiments to evaluate candidate long-range DNA interactions in the 16p13 region. This indicated the mechanism by which disease-associated SNPs within one gene, CLEC16A, might influence the expression of the neighbouring gene, DEXI.

RESULTS

eQTL analysis in human monocytes

The two independent data sets interrogated were generated previously from normal human monocytes purified from fresh blood samples from 1370 individuals [Gutenberg Health Study (GHS) (16)] and 753 individuals [Cardiogenics Project (CGP) (17), http://www.cardiogenics.eu/web/], respectively, and subjected to genome-wide genotyping and microarray gene expression analysis. In both data sets, we found evidence of a single eQTL in the region. Expression of the DEXI gene was correlated with several chromosome 16p13 SNPs in high LD within CLEC16A, including rs12708716 (Fig. 1) and rs8062322. Since the majority of the DEXI eQTL SNPs in intron 10 and intron 19 of the CLEC16A gene are in high LD, a single SNP most correlated with DEXI expression could not confidently be determined. The CLEC16A alleles that confer protection from T1D and MS were only associated with increased expression of DEXI (P = 3.8 × 10−38 in GHS data set; P = 1.8 × 10−7 in CGP data set), and not with expression of any other genes in the region, in both monocyte expression data sets.

Importantly, there was no correlation between expression of DEXI and SNPs in the proposed second region of T1D association, near C16orf75 (P > 0.05). In addition, we noted specifically that no eQTLs were detected for expression of any other gene within the 16p13 region, including CLEC16A and SOCS1 in either data set (Supplementary Material, Fig. S1). In addition, in contrast to a recent report evaluating gene expression in the 16p13 region in lymphoblastoid cell lines, no correlation between expression of SOCS1, DEXI and CLEC16A was detected (6).

We identified confirmatory evidence supporting an eQTL within intron 19 of CLEC16A for DEXI expression within the supplementary information of two further, recent independent data sets (18,19). The first was an investigation of eQTLs in Epstein Barr virus (EBV)-transformed lymphoblastoid cell lines (18), and the second, a study of eQTLs in human primary bone cells treated with PGE2 (19). Both studies correlated DEXI expression by microarray (Supplementary Material, Fig. S2), with genotype at eQTL SNPs within intron 19 of CLEC16A, and neither study reported eQTLs in the 16p13 region for expression of CLEC16A, SOCS1 or CIITA.

Evaluation of candidate long-range DNA interactions by 3C

The DEXI promoter region and the SNP most associated with T1D in CLEC16A (rs12708716) are separated by a distance of ~160 kb. If one region is to influence the other, this would suggest that a DNA loop is formed during transcription of DEXI, allowing the two regions to be in close physical proximity. The 3C technique has been used to examine long-
Expression of the DEXI gene, contained within the 16p13 region of T1D association (A) by microarray using Illumina probe set ILMN_1738866, is correlated with the genotype at SNPs within the CLEC16A gene in two independent data sets. The first data set (i) was generated from purified human monocytes \((n = 1370\) individuals) collected during GHS and the second (ii) from purified human monocytes \((n = 753\) individuals) collected during the CGP Project. SNPs associated with T1D are illustrated on the same scale (iii) to illustrate the co-localization of the eQTL and T1D association signals. Boundaries of intron 10 and intron 19 of CLEC16A contain the most associated SNPs for T1D and are contained within orange and black dotted lines, respectively. (C) DEXI expression by genotype at the T1D-associated SNP rs12708716 within the published GHS monocyte data set, where the minor (G) allele is protective, and (D) DEXI expression by genotype at the T1D-associated SNP rs725613 in the unpublished CGP monocyte data set.

**Figure 1.**
distance chromosomal interactions (20,21) and was employed here to test our hypothesis that the DEXI promoter region and intron 19 of CLEC16A are in close physical proximity during gene transcription. The BglII restriction enzyme was chosen for use in 3C since it makes two cuts in the 2 kb region between the promoter regions of CLEC16A and DEXI, allowing fragments containing these regions to be evaluated separately, and it also cuts seven times throughout intron 19 of CLEC16A. Three human cell lines were selected for these experiments—a monocyte cell line (THP-1), since we identified the eQTL originally in monocytes, a lung epithelial cell (A549), since DEXI expression was first reported in this cell line (22) and a human EBV-transformed B-cell line, since evidence exists for the DEXI eQTL in CLEC16A in this cell type in the literature (18).

The 3C technique generates ligated DNA fragments through cross-linking of distal DNA sequences based on their physical proximity in living cells. Using a ‘bait’ reverse primer next to a BglII restriction site located in the region of the DEXI promoter, quantitative polymerase chain reactions (qPCRs) were initially undertaken using 13 different forward primers in candidate interaction BglII fragments, throughout the 16p13 region. In all three cell lines, a specific association was detected between a fragment representing the DEXI promoter region and a single specific BglII fragment of ~20 kb within intron 19 of CLEC16A (Figs 2 and 3). This finding was confirmed by gel electrophoresis and direct sequencing of the qPCR products generated (data not shown). The additional interaction detected between the DEXI promoter fragment and an adjacent fragment containing the CLEC16A promoter region is related to the proximity of these two regions in the genome, and reflects random collisions between the chromatin fibres, commonly seen in 3C experiments (23).

The interaction between the DEXI promoter region and intron 19 of CLEC16A was confirmed using several different primer sets. First, using alternative DEXI promoter primers and a more comprehensive set of primer pairs for BglII sites within intron 19 of CLEC16A, the original region of interaction was confirmed as the only interacting fragment within intron 19. Second, reciprocal qPCRs were undertaken using a primer in the interacting intron 19 fragment as ‘bait’. As well as confirming the interaction with the DEXI promoter, these experiments also demonstrated that this specific region of intron 19 did not interact with any other candidate sites (Supplementary Material, Table S1) within the region. We also conducted further 3C/qPCR analysis of potential interactions involving intron 10 BglII fragments, to evaluate the possibility of a double loop, involving both intron 10 and intron 19 of CLEC16A since disease-associated SNPs have also been reported within intron 10. We used both the DEXI promoter region and the interacting region of intron 19 as bait, but found no evidence of further interactions by qPCR (data not shown). Finally, additional qPCRs were undertaken using the interaction region in intron 19 as a ‘bait’ to test for DNA interactions within intron 19 itself. No further sequencing-confirmed interactions were detected in any of these experiments (data not shown), but the qPCR product representing the DEXI promoter region—intron 19 interaction was consistently present.

The challenge of progressing from a disease-associated region identified in GWAS to convincing evidence for which gene(s) are causal candidates in the disease pathogenesis is considerable. This is, in part, due to the fact that a large region of LD can contain many genes, in addition to the strong possibility that the causal variant, lying within the LD region, affects the expression of genes outside the LD block in long-range functional interactions. We note that rs12708716, reported in several studies as the SNP most associated with T1D and MS, and shown here to correlate with DEXI expression, lies within the 20 kb fragment that we have shown to interact with the DEXI promoter region (Fig. 3).

Evaluation of epigenetic markers associated with enhancer activity and transcription factor binding

Non-coding regions of DNA may impact upon expression of distant genes by acting as enhancers, requiring looping of DNA and interaction of remote regions via protein–protein contacts (24). Although the exact mechanisms by which enhancers or suppressors mediate their effects on gene transcription are unknown, many enhancer regions contain binding sites for transcription factors and are identifiable by the presence of epigenetic histone modifications such as H3K4me1 and H3K27Ac (25,26). In addition, recent evidence suggests that some enhancers in non-coding parts of the genome are transcribed, by RNA polymerase II, into RNA with regulatory functions (27–29). We, therefore, made use of publicly available genome-wide ChIP-Seq data sets from the ENCODE project (30,31) to
investigate histone modification marks and RNA polymerase binding throughout the 16p13 region and found intron 19 of CLEC16A to be particularly enriched for enhancer marks in human cells (Supplementary Material, Fig. S3A). Further evidence for a regulatory function of intron 19 of CLEC16A is provided by the observation that its sequence is highly conserved.
between mouse and humans, consistent with an important regulatory function in both species. It is bound by multiple transcription factors in a murine haematopoietic progenitor cell line (32) (Supplementary Material, Fig. S3B) and human cell lines (N.K.W., unpublished data). Many of these transcription-factor-binding events occur towards the 3’ end of the fragment that we have shown to interact with the DEXI promoter region in human cells. We also note that, according to ENCODE ChIP-Seq data from human cell lines, both the interacting region in intron 19 and the DEXI promoter region share binding sites for several transcription factors (Fig. 3A and B) including NF-κB, JunD and c-Myc. Furthermore, as might be predicted by recent work (29), clear RNA-polymerase-II-binding peaks are evident within the fragment of intron 19 of CLEC16A containing the putative enhancer region (Fig. 3B).
The gene expression and 3C data provide strong support for DEXI’s candidacy as a new autoimmunity gene. Apart from a pseudogene on chromosome 15, which we have shown to produce little or no RNA (Supplementary Material, Fig. S4), DEXI has no clear parologue in the human genome. Although CLEC16A itself appears to be the focus of many investigators studying the 16p13 region, our results imply an immediate additional need to investigate and understand the biology of DEXI and its role in the development of autoimmune disease.

Figure 3. The interaction sites detected by 3C at the DEXI promotor region (A) and intron 19 of CLEC16A (B) are enriched for transcription-factor-binding sites (marked by grey and black horizontal lines) and enhancer-associated histone modifications (ENCODE project, displayed using the UCSC Genome browser). Intron 19 of CLEC16A also contains RNA polymerase II binding peaks. The 3C interaction fragment, between two BglII restriction enzyme digestion sites, is highlighted with a yellow line.
DISCUSSION

This study highlights DEXI as an autoimmune candidate gene in 16p13, based on an eQTL for DEXI in monocytes that appears to co-localize with the T1D-association signal and demonstration of close physical proximity of regulatory sequences in intron 19 of CLEC16A with the DEXI promoter region by the 3C technique. DEXI was originally identified in 2001 as a transcript which was differentially expressed in lung tissue of patients with emphysema compared with normal lung tissue (22). The function of DEXI is unknown; however, the gene was named dexamethasone-induced transcript since dexamethasone treatment of the A549 cell line, used here for the 3C experiments, was reported to increase DEXI mRNA expression using a semi-quantitative blotting method. More recently reported RNA-Seq and microarray results using dexamethasone-treated cell lines do not support this claim (19,33) and, therefore, we suggest that this name may be a misnomer.

Historically, eQTLs have been classified into cis and trans (or distant), where cis eQTLs affect expression of a gene nearby and trans act at greater distances (usually on another chromosome) in a more indirect way, such as affecting the expression of a regulator that has a subsequent impact of the trans-controlled gene. The findings presented here represent a biomedically important finding at an intermediate distance level, rather than a classical cis or trans effect. The eQTLs act on the same chromosome, but at a distance of >150 kb, and importantly do not, according to our current results, affect the nearest gene, but the next one along chromosome 16p13. It is likely that this will not be an isolated event in future studies of common disease, hence this finding is very relevant to the wider field of GWAS follow-up. In addition, although the interaction fragment in intron 19 contains several disease-associated SNPs, the question of exactly how allele-specific expression is driven is raised, and how this process is made gene-specific. Since the other genes in the region are expressed, their promoters are accessible, so it is unlikely that specificity is simply related to promoter accessibility. Instead, we hypothesize that specific transcription factor combinations bound to the intron 19 region and DEXI promoter are important and the mechanism by which gene expression is controlled might involve transcription of intron 19 and its function as a non-coding regulatory RNA. Recent evidence of a strong genetic component for allele-specific differences at the level of transcription factor binding and chromatin structure has been reported (34). In addition, the recruitment of cohesin and the multi-protein Mediator complex has been associated with chromosome looping and regulation of gene expression via enhancer sequences (35). We postulate that the causal variant(s) in the region affect enhancer activity and that this could arise because of an allele-specific effect on chromatin structure and/or recruitment of a multi-protein transcriptional co-activator complex (Fig. 4).

Our study illustrates that increased expression of DEXI in monocytes is associated with SNP alleles that are protective from autoimmune disease. Monocytes are already known to be important in the pathogenesis of T1D, giving rise to macrophages, and the dendritic cells that are central in ‘priming’ the islets and establishing an inflammatory milieu prior to the destruction of islets by antigen-specific T cells (36). In addition, monocytes, macrophages and microglia have a central role in the central nervous system (CNS) inflammation of MS. During MS attacks, T lymphocytes and monocyte-derived macrophages gain entry to the CNS and form peri-vascular infiltrates, a process which is accompanied by enhanced permeability of the blood–brain barrier (37,38). Although monocytes play a role in disease risk, we note that the DEXI transcript is found in higher abundance in CD4+ and natural killer cells compared with monocytes, and additional studies have provided evidence for a DEXI eQTL in intron 19 of CLEC16A in cells other than monocytes, including primary bone cells and EBV-transformed lymphoblastoid cell lines (18,19). Therefore, it is possible that DEXI’s role in autoimmunity is related to genotype-regulated expression in a cell subset other than, or in addition to, monocytes and might also be specific to a particular time in development. We note that DEXI expression was increased in macrophages, generated in culture from a subset of the CGP samples, compared with resting monocytes (Supplementary Material,
Fig. S5), but that the DEXI eQTL was not preserved in macrophages (data not shown). We postulate that this may be the result of a stricter (genotype-related) control of basal expression in monocytes.

The DEXI gene is conserved across many species including the mouse, rat, dog, elephant, zebrafish and chicken, but is not found in Caenorhabditis elegans or Drosophila (Supplementary Material, Fig. S6). It is predicted by some, but not all, software packages to contain a trans-membrane domain, a sequence with a repeating leucine motif (Supplementary Material, Fig. S6) and a predicted casein kinase phosphorylation site (22). Expression is most strongly detectable by microarray in liver, brain, heart and lung tissue, as well as in some cells of the immune system (http://biogps.gnf.org/#goto=generereport&id=28955 and Supplementary Material, Fig. S7). Although not originally an obvious T1D candidate gene at 16p13, a previous survey of allelic expression using EST mining lists DEXI as one of 40 genes whose expression is in allelic imbalance by in silico analysis (39). It is also interesting to note that DEXI expression is reported to be affected by exposure to cigarette smoke (16) (P = 8 × 10⁻⁸, data not shown), perhaps explaining in part the original report of increased DEXI expression seen in the lungs of emphysema patients (22). Additional opportunities for the control of DEXI expression within cells exist at the level of microRNAs, since binding sites for microRNAs, including miR-137 and miR-30–5p, are predicted in the 3'UTR of the gene, using TargetScan (40).

To our knowledge, this is one of only a small number of reports of successful application of 3C to the dissection of GWAS SNPs and target genes (41–44). In contrast to previous publications, however, the present study is the first to report GWAS SNP alleles within an intron region appearing to regulate expression of a neighbouring gene. These data provide an obvious rationale to search for long-distance regulatory sequences as an explanation for a proportion of GWAS SNP associations. The experiments reported here were able to identify a long-distance interaction using 3C, based on a hypothesis generated using eQTL data. Further evaluation of the region using more exhaustive interrogation methods for interacting fragments in a variety of tissues may yet yield evidence of further interactions. Taken together, there is now clear justification not only for the evaluation of the mechanism(s) by which DEXI might protect against autoimmune disease, but also for the development and use of 3C techniques involving next-generation sequencing, on a genome-wide scale, such as ChIA-PET (45), HiC (46) and 5C (47) in multiple cell types. Based on the findings presented here, we propose that the small DEXI gene represents the key to a novel and important pathway in the pathogenesis of T1D, MS and other immune-mediated diseases.

**Materials and Methods**

**Ethics Statement**

All research involving human participants has been approved by Fenland and Peterborough Local Research Ethics Committee.

**Monocyte purification and preparation for microarray**

*Gutenberg Health Study*. This protocol has been previously described (16,17) but briefly, GHS is a community-based, prospective, observational single-centre cohort study in the Rhein-Main region in Germany. Separation of monocytes was conducted within 60 min of collection of 8 ml of blood in patients recruited as part of this study, and RNA was extracted the same day. Monocytes were separated by negative selection, using the Vacutainer CPT Cell Preparation Tube System (BD, Heidelberg, Germany) with 400 µl Rosette Sep Monocyte Enrichment Cocktail (StemCell Technologies, Vancouver, Canada) added immediately after blood collection. After separation, cells were washed twice in ice-cold phosphate-buffered saline (PBS) buffer containing 2 mM EDTA and the purity in selected samples was confirmed by flow cytometry. Cells were resuspended in 1.5 ml TRIzol reagent (Invitrogen, Karlsruhe, Germany) and RNA extraction was performed within 5 h using chloroform extraction and the RNase Mini Kit (Qiagen, Hilden, Germany), including DNase digestion. Genome-wide expression analysis was performed on monocyte RNA samples using the Illumina HT-12 v3 BeadChip (http://www.Illumina.com), in batches of 96 samples. Genotyping was performed using the Affymetrix (Santa Clara, CA, USA) Genome-Wide Human SNP Array 6.0 and the Genome-Wide Human SNP NspI/Styl 5.0 Assay Kit.

**Cardiogenics project**. This protocol has previously been described (17), but briefly, the multi-centre Cardiogenics (CGP) study includes 363 patients with coronary artery disease or myocardial infarction and 395 healthy individuals of European descent (http://www.cardiogenics.eu). Blood samples (30 ml) from fasting subjects were collected into EDTA blood tubes and monocytes were isolated by positive selection, using CD14 microbeads and AutoMACS/Auto-MACS Pro (Miltenyi - Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Monocyte purity was measured as the percentage of CD14⁺ve cells analysed by flow cytometry. Isolated monocytes were lysed in TRIzol reagent and RNA was extracted by a method similar to that described above for GHS. Whole-genome genotyping was carried out at the Wellcome Trust Sanger Institute, using two arrays, the Sentrix Human Custom 1.2M array and the Human 610 Quad Custom array (Illumina). Gene expression profiling was performed using Human Ref-8 Sentrix Bead Chip arrays (Illumina).

**eQTL analysis**

Normalized expression probes in 16p13 were tested for association in the region, assuming an additive model, using the ‘R’ software package.

**3C and qPCR**

The method for 3C has been previously reported in detail (21) by co-authors N.F.C. and P.F., so is described briefly here. The lung epithelial cell line A549 and the monocyte-like cell line THP-1 were obtained from ATCC (http://www.lgcstandards-atcc.
org/ATCC Cultures and Products/Cell Biology/) and grown according to standard tissue culture protocols, in addition to an EBV-transformed B-cell line from the CEPH collection obtained from ECACC (http://www.hpacultures.org.uk/collections/ecacc.jsp).

Fifty million cells at ~80% confluence from each cell line were harvested and washed in ice-cold PBS before being passed through a cell strainer. Following a second PBS wash, cells were re-suspended in 45 ml high-glucose DMEM medium + 1% glutamine (Invitrogen) and 10% fetal bovine serum. Cross-linking was performed by adding 2.7 ml of 37% formaldehyde (Merck) for 5 min at room temperature with mixing. The reaction was quenched with 3 ml of 2 M glycine, followed by washing with 50 ml ice-cold PBS. The pellet for each cell line was re-suspended in 50 ml of permeabilization buffer [10 mM Tris–HCl, pH 8, 10 mM NaCl, 0.2% igepal and one complete protease inhibitor tablet, EDTA-free (Roche Diagnostics, Penzberg, Germany)] and incubated at 4°C on ice with mixing. Following a centrifugation step, and manual cell count, each 1 × 10⁷ nuclei from each cell line were resuspended in 100 µl of NEB Buffer 3 (New England BioLabs, Ipswich, MA, USA). One 1 × 10⁷ nuclear pellet from each cell line was treated with 7.5 µl of 20% SDS for 1 h at 37°C, 950 r.p.m. on a Thermomixer (Eppendorf, Histon, UK), to remove any non-cross-linked proteins from the DNA. This was followed by the addition of 50 µl of 20% Triton-X100 (Sigma-Aldrich) for 1 h at 37°C, 950 r.p.m., to sequester SDS and allow subsequent digestion. BgIII enzyme (1500 IU) (NEB) was added to each tube, followed by incubation overnight at 37°C and 950 r.p.m.

A 5 µl of aliquot of digested DNA was assessed for completeness of digestion using gel electrophoresis. The BgIII enzyme was inactivated by the addition of 40 µl of 20% SDS for 25 min at 65°C at 950 r.p.m. The incubated samples were added to 7 ml of 1.1× ligation buffer (NEB) and 375 µl of Triton-X100 for 1 h at 37°C, with mixing. The cross-linked digested DNA was re-ligated by the addition of 800 IU NEB T4 DNA ligase for 4 h at 16°C and then 30 min at room temperature. Then 900 µg of Proteinase K (Roche Diagnostics) was added followed by an overnight incubation at 65°C.

The samples were cooled to room temperature and 300 µg of RNAse A (Sigma) added for 1 h at 37°C. This was followed by phenol–chloroform extraction and ethanol–acetate precipitation of DNA, which was quantified by PicoGreen assay.

3C interaction products were detected by PCR using candidate primer pairs with and Qiagen HotStar Taq Polymerase and 250 ng of DNA per reaction, followed by agarose gel electrophoresis. Quantification of interaction products was undertaken using qPCR and 2× SYBR Green Mastermix (Applied BioSystems, Final 1×) and candidate forward and reverse primers at a final concentration of 400 nM. Samples were tested in a 96-well format in triplicate, using the ABI 7000 sequence detection system. Quantification was achieved using serial dilutions of a 3C-positive control template on each plate. This was generated by synthesis of all possible PCR products using the available primers (Supplementary Material, Table S1), followed by gel extraction and purification. PCR products underwent BgIII digestion before being mixed in equimolar concentrations and ligated with T4 ligase (NEB) to generate a pool of potential interaction products, which was purified by phenol–chloroform extraction and ethanol precipitation. The control template was mixed with genomic DNA that had undergone digestion and random ligation so that PCR efficiency was not affected by the total amount of DNA present (similar to the 300 ng/reaction for the real 3C samples) (16). Quantification of PCR products was achieved by comparison with the standard curve on each plate and in addition, values were normalized for each experiment using the result obtained from the most 3’ interacting primer and the bait within each cell line.

Sequencing of qPCR products at candidate interaction sites was undertaken following gel purification and PCR of purified products with the original 3C primers and the BigDye Terminator v3.1 sequencing kit. Samples were analysed in triplicate from each cell line, using the 3730xl DNA Analyzer (Applied BioSystems) and sequencing traces were visually checked using the Peak PickerTM software.

AUTHORS’ CONTRIBUTIONS
Access to GHS and CGP data was provided by S.B. and F.C., with the written permission of their respective data management groups. eQTL and co-localization analysis was developed and performed by C.W. and J.D.C.

L.J.D., C.W. and J.A.T. participated in the conception and overall design of the study. The manuscript was prepared by L.J.D., J.A.T. and J.D.C. with helpful comments from N.F.C., P.F., B.G., F.C., S.B., J.M.M.H., D.J.S., N.K.W. and P.F. Supplementary Material, Figure S4b was prepared by N.K.W.

3C experiments were designed by L.J.D., J.A.T., N.F.C., P.F., N.K.W. and B.G. and performed by L.J.D., with the guidance of N.F.C. and assistance with tissue culture from S.D.

DNA collection, preparation and curation were undertaken by S.N., D.J.S., H.E.S. with database management by N.M.W. and statistical assistance by J.M.M.H.

S.B., T.Z., T.M. and K.L. participated in the Gutenberg Health Study.

F.C. and C.M.R. participated in the Cardiogenics Study.

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Quantitative PCR for DEXI and PseudoDEXI was undertaken by L.J.D., K.L.A., N.S. and A.A.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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