Recessive mutations in a distal *PTF1A* enhancer cause isolated pancreatic agenesis

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The contribution of cis-regulatory mutations to human disease remains poorly understood. Whole-genome sequencing can identify all noncoding variants, yet the discrimination of causal regulatory mutations represents a formidable challenge. We used epigenomic annotation in human embryonic stem cell (hESC)-derived pancreatic progenitor cells to guide the interpretation of whole-genome sequences from individuals with isolated pancreatic agenesis. This analysis uncovered six different recessive mutations in a previously uncharacterized ~400-bp sequence located 25 kb downstream of *PTF1A* (encoding pancreas-specific transcription factor 1a) in ten families with pancreatic agenesis. We show that this region acts as a developmental enhancer of *PTF1A* and that the mutations abolish enhancer activity. These mutations are the most common cause of isolated pancreatic agenesis. Integrating genome sequencing and epigenomic annotation in a disease-relevant cell type can thus uncover new noncoding elements underlying human development and disease.

Most individuals with syndromic pancreatic agenesis have heterozygous dominant mutations in *GATA6* (refs. 1,2). Extrapancreatic features in these individuals include cardiac malformations, biliary tract defects, and gut and other endocrine abnormalities. Four families have been reported with syndromic pancreatic agenesis, with severe neurological features and cerebellar agenesis caused by recessive coding mutations in *PTF1A*\textsuperscript{1–5}. Most cases of isolated, non-syndromic pancreatic agenesis remain unexplained, with the only cause described being recessive coding mutations in *PDX1* that were reported in two families\textsuperscript{6,7}. We previously noted that individuals with unexplained pancreatic agenesis were often born to consanguineous parents and rarely had extrapancreatic features\textsuperscript{1}. These observations suggested an autosomal recessive defect underlying isolated pancreatic agenesis.

To identify recessive mutations causing isolated pancreatic agenesis, we used linkage and whole-genome sequencing analyses. Initially, we performed homozygosity mapping in six affected subjects and one unaffected subject from three unrelated consanguineous families (Supplementary Fig. 1). This analysis highlighted a single shared locus on chromosome 10 that included *PTF1A*, but mutations in the coding and promoter sequences of *PTF1A* and in the coding sequences of 24 other genes in the region were excluded by Sanger sequencing (Supplementary Fig. 1 and Supplementary Table 1). We next performed whole-genome sequencing of probands from the two families with multiple affected individuals. We first looked for homozygous coding mutations in the exomes of the two individuals for whom whole-genome sequencing was performed. Each genome contained ~3.6 million variants, from which we filtered out any that were present in 81 control genomes or that were present at a frequency of >1% in 1000 Genomes Project data\textsuperscript{8}. This filtering left 2,868 and 3,188 rare or newly identified homozygous single-nucleotide variants (SNVs) and indels per subject. Of these, 8 and 19 per subject were annotated as missense, nonsense, frameshift or essential splice site (Supplementary Table 2). However, these coding variants either did not cosegregate with disease or were not considered plausible candidates for having a role in pancreas development (Supplementary Table 2).

We next searched for noncoding disease-causing mutations among the remaining candidate homozygous variants. We reasoned that any causal variant should disrupt a noncoding genomic element that is active in cells that are relevant to this disease. As isolated pancreatic agenesis must be the result of a defect in early pancreas development, we determined whether any of the rare or newly identified

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homozgy variants in these subjects mapped to active regulatory regions in pancreatic endoderm cells derived from hESCs (Fig. 1 and Supplementary Figs. 2 and 3). We thus defined 6,109 putative transcriptional enhancers in embryonic pancreatic progenitors that were enriched in monomethylation of histone H3 at lysine 4 (H3K4me1), a post-translational histone modification that is associated with enhancer regions, and were also bound by two or more pancreatic developmental transcription factors that are known to be essential for early pancreas development. Seven homozgy variants from each subject occurred in one of these annotated noncoding regions. However, only 1 of the 6,109 regulatory regions contained a variant in both sequenced individuals, and this was the same variant in one subject with pancreatic agenesis and intrahepatic cholestatic failure from which he died. Filled black symbols represent individuals with isolated pancreatic agenesis. The striped symbol represents an individual with extrahepatic features. Filled gray symbols represent individuals with pancreatic agenesis and intrahepatic cholestasis.

Figure 1 Epigenome annotation of variants from genome sequencing identifies a shared variant in a putative enhancer element. A variant identified by whole-genome sequencing and five additional variants identified in subjects with pancreatic agenesis map to a 25-kb region downstream of PTF1A, which contains a single candidate pancreatic progenitor-specific enhancer in a highly conserved 400-bp element. The top panel shows chromatin immunoprecipitation and sequencing (ChIP-seq) density plots for the enhancer mark H3K4me1, and the second and third panels show occupancy for FOXA2 and PDX1, respectively. A broad panel of embryonic and adult human tissues do not show active chromatin marks in this region (Supplementary Fig. 5). Vertebrate conservation and mammalian conservation tracks (with conservation measured by GERP score) show the high conservation of this element. The red line depicts the approximate location of a 7.6-kb deletion in this region, and red arrows indicate the positions of point mutations on chromosome 10.

We sequenced this putative pancreatic developmental enhancer in 19 additional probands with pancreatic agenesis of unknown etiology (9 with extrapancreatic features and 10 isolated cases) and identified recessive mutations in 7 of the 10 individuals with non-syndromic pancreatic agenesis. We also identified a homozgyous mutation in one subject with pancreatic agenesis and intrahepatic cholestasis failure (Fig. 2 and Supplementary Table 3). Of the ten probands with mutations in this element, six had the same chromosome 10 mutation, g.23508437A>G, as part of a shared extended haplotype (minimal shared haplotype of 1.2 Mb; Supplementary Fig. 6). Three of the remaining probands had different base-substitution mutations on chromosome 10: a homozgyous g.23508363A>G mutation, a homozgyous g.23508305A>G mutation and compound heterozygous g.23508365A>G; [23508446A>C] mutations (Fig. 2). In the tenth family, we identified a 7.6-kb deletion by long-range PCR, and sequence analysis showed that the deleted region (chr. 10: 23,502,416–23,510,031) included the entire putative enhancer (Supplementary Fig. 7).

Testing of parents and siblings demonstrated cosegregation of the mutations with diabetes and exocrine insufficiency (Fig. 2 and Supplementary Table 3). None of the mutations were present in 1,092 individuals from the 1000 Genomes Project4 or in dbSNP137, and Sanger sequencing of 299 controls did not detect any of these variants. The deletion

Figure 2 Families with mutations in the PTF1A enhancer. Filled black symbols represent individuals with isolated pancreatic agenesis. The striped symbol represents an individual who had pancreatic agenesis with intrahepatic cholestasis failure from which he died. Filled dark gray symbols represent individuals with exocrine insufficiency and young-onset diabetes (age at diagnosis <22 years). Whole genome–sequenced individuals are indicated by asterisks. DNA from the parents in families 2, 5, 6 and 8 was not available. Del, deletion; N, no deletion.
Pancreas agenesis–associated mutations disrupt the function of a transcriptional enhancer that is specifically active in pancreatic progenitors. (a) Reporter assays for the new enhancer in hESC-derived pancreatic endoderm progenitors, several adult pancreatic exocrine transformed cells (Panc-1, 266-6 and AR42J) and HeLa cells. Transcriptional enhancer activity was only observed in hESC-derived pancreatic progenitor cells, and this activity was disrupted by all five mutations. Asterisks indicate Student’s t test P < 0.05 for comparisons of mutant and wild-type enhancers in progenitors. Error bars, s.d. from three independent experiments. (b) 3C analysis shows that the newly identified enhancer interacts directly with the PTF1A promoter in hESC-derived pancreatic progenitor cells. The viewpoint at the PTF1A enhancer (E) is indicated by an arrow, and the approximate regions that were tested for interaction with the viewpoint (C1, C2, C3 and P for the PTF1A promoter) are shown in e. C4 represents a control region in an unrelated locus. Error bars, s.d. from three independent experiments. (c) Pancreas agenesis–associated mutations target critical residues in predicted binding sites for the pancreatic regulators FOXA2 and PDX1. (d) Electrophoretic mobility shift assay showing high-affinity, sequence-specific interaction of FOXA2 with a double-stranded oligonucleotide containing the wild-type (WT) sequence but not the g.23508363A>G mutation (Mut). The retardation signal was suppressed by unlabeled consensus high-affinity binding site for FOXA2 and was supershifted with antibodies recognizing FOXA2. Black triangles represent competition gradients of 30- and 100-fold excess cold probe. Binding activity is shown for MIN6 cells and dissected pancreatic buds from embryonic day (E) 12.5 mouse embryos. Probe refers to the radioactively labeled probe used in binding assays. The arrow indicates the FOXA2 retardation complex. (e) Electrophoretic mobility shift assay showing sequence-specific interaction of PDX1 with oligonucleotide containing the wild-type (WT) sequence but not the g.23508446A>C mutation (Mut). The retardation signal was suppressed by unlabeled consensus high-affinity binding site for PDX1 and was supershifted with antibodies recognizing PDX1. Competition was performed with 100-fold excess cold probe. Binding activity is shown for MIN6 cells and pancreatic buds from E11.5 mouse embryos. The arrow indicates the PDX1 retardation complex.

whereas the remaining point mutation disrupted the affinity of an uncharacterized sequence-specific DNA-binding protein present in mouse pancreatic progenitors (Fig. 3d,e and Supplementary Fig. 8). Notably, all five mutations disrupted the enhancer activity of this region in hESC-derived human pancreatic progenitors (Fig. 3a). Collectively, these findings show that multiple mutations causing isolated pancreas agenesis disrupt the function of a previously unrecognized enhancer that targets PTF1A in human embryonic pancreatic progenitor cells.

The contribution of noncoding variants to human disease remains poorly understood. There are examples of mutations in distal regulatory elements causing monogenic disease11–14, but the number of such mutations is small compared to that for coding mutations. Although whole-genome sequencing technologies allow the identification of all genetic variation, the discrimination of functional noncoding causal variants among the millions of noncoding variants present in each individual remains a formidable challenge. The ENCODE Project has recently uncovered functional elements throughout the noncoding genome, leading to expectations that annotation of these elements can be integrated with genome sequencing to discover causal noncoding mutations15. Our study now provides an example that validates this expectation and shows that recessively inherited distal cis-regulatory mutations in a newly identified developmental enhancer are the most common cause of a rare mendelian disease. The fact that the mutated regulatory element was exclusive to embryonic pancreatic progenitors out of a broad panel of adult and embryonic tissues highlights the importance of analyzing diseaserelevant genomic annotations. Our results support efforts to identify mutations in regulatory elements in monogenic disorders by integrating genome sequencing.
data with functional annotation from projects such as ENCODE\textsuperscript{15} and the Epigenome Roadmap\textsuperscript{16}. These findings may also be relevant for future efforts to discover causal alleles in common non-mendelian diseases, where many susceptibility variants appear to lie outside coding regions\textsuperscript{17}.

In summary, we have demonstrated that mutation of a new distal developmental enhancer of PTF1A is a common cause of isolated pancreatic agenesis in humans, and we demonstrate the potential of integrating genome sequencing with epigenomics to identify mutations in new regulatory elements that cause disease.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** All ChIP sequencing data from this study have been deposited in ExpressArray under accession E-MTAB-1990.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

M.N.W., S.E., J.F and A.T.H. designed the study. M.N.W., A.-M.P., J.A.L.H. and H.L.A. performed bioinformatic analyses. I.C., S.A.R.-S., C.H.-H.C., A.M., L.V. and J.F performed functional studies. A.-M.P., J.A.L.H., E.D.F., R. Caswell, S.E.F. and S.E. performed Sanger sequencing or deletion analysis and interpreted the results. S.F.F., C.S.-S., K.H., C.L.R., R. Chen, P.M. and A.T.H. analyzed the clinical data. M.N.W., I.C., A.-M.P., S.E., J.F. and A.T.H. prepared the draft manuscript. All authors contributed to discussion of the results and to manuscript preparation.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Determination of pancreatic endoderm from human ESCs. Human ESCs (H9, WiCell) were imported under the guidelines of the UK Stem Cell Bank Steering Committee (authorization SCCS10-44). Cells were maintained and differentiated into artificial pancreatic progenitors using a protocol that was previously fully described. These artificial pancreatic progenitors express a constellation of pancreatic endoderm markers including PDX1, HLXB9, NKX6.1, SOX9, HNF6 and PTF1A. In brief, definitive endoderm was induced by growing hESCs in Advanced DMEM (Invitrogen) supplemented with SB-431542 (10 µM) and were grown for 3 more days in Advanced DMEM supplemented with 1% B27. For filtering SNVs and indels, we used 69 publically available whole genomes provided by Complete Genomics and 12 additional whole genomes that had also been sequenced by Complete Genomics for a non-overlapping data set. We also filtered out variants present at minor allele frequency of >1% in the 1000 Genomes Project.

ChIP-seq maps of pancreatic progenitor regulatory elements. Chromatin immunoprecipitation for H3K4me1 (Abcam, ab-8895; n = 2), FOXA2 (Santa Cruz Biotechnology, sc-9055X; n = 1), HNF1β (Santa Cruz Biotechnology, sc-22840-X; n = 1), ONECUT1 (Santa Cruz Biotechnology, sc-13050; n = 1) and PDX1 (BCBC, AB2027; n = 1) was performed essentially as described previously. For the stage (days 7–10), cells were cultured in Advanced DMEM supplemented with human FGFI10 (50 ng/ml; AutobioBiocare), all-trans retinoic acid (2 µM; Sigma) and Noggin (150 ng/ml; R&D Systems) for 3 d (days 4–6). For the next stage (days 7–10), cells were cultured in Advanced DMEM supplemented with human FGFI10 (50 ng/ml; AutobioBiocare), all-trans retinoic acid (2 µM), KAAD-cyclopamine (0.25 µM; Toronto Research Chemicals) and Noggin (150 ng/ml) for 3 d. For the last stage (days 10–12), cells were cultured in Advanced DMEM supplemented with human FGFI10 (50 ng/ml; R&D Systems) for 3 d. For matura-

Conservation analysis. Eighty percent of bases on chromosome 10 between positions 23,508,149 and 23,508,510 were classified as being part of a vertebrate conserved element by PhastCons (logarithm of odds (LOD) > 17). Multiz alignment results obtained from the UCSC Genome Browser showed that there is conservation of the entire element from human to chicken and that over half the element is conserved to Xenopus tropicalis. All mutated bases are highly conserved, with all having GERP scores of 5.65.

Sanger sequencing of the PTF1A element. We amplified the conserved 400-bp element using the primers listed in Supplementary Table 4. PCR products were sequenced on an ABI3730 capillary machine (Applied Biosystems) and analyzed using Mutation Surveyor v3.98 (SoftGenetics).
were isolated for library preparation with NextFlex adaptors with a 6-base index sequence tag set. Individual libraries were enriched by six cycles of PCR amplification and were then pooled in equimolar quantities for 100-bp paired-end sequencing on an Illumina HiSeq 2000 sequencer. We used the Burrows-Wheeler Aligner (BWA v0.6.2) to align sequence reads to the hg19 reference genome and then visualized the breakpoints using the Integrative Genomics Viewer, which demonstrated that the deletion breakpoints occurred at positions 23,502,416 and 23,510,031 on chromosome 10 (Supplementary Fig. 7). The deletion mutation was investigated in all available members of family 7 using a junction fragment PCR assay (primer sequences available upon request).

Sanger sequencing of the PTF1A enhancer in control samples. We sequenced the putative PTF1A enhancer element in 150 healthy controls of European descent from the Exeter Family Study of Childhood Health and in 149 individuals from Turkey using Sanger sequencing (Supplementary Table 4). No variants were identified.

Transcription factor binding motif analysis. Motif discovery over the point mutation sites comparing wild-type and mutation-containing sequences was performed using HOMER.

Electrophoretic mobility shift assays. Mouse pancreatic ducts were dissected from E11.5 and E12.5 C57BL/6J mouse embryos as described. The study was approved by the Animal Experimentation Ethics Committee of the University of Barcelona. Nuclear extracts were purified as described. Binding of nuclear extracts from embryonic pancreas and MIN6 β cells (kindly provided by J.I. Miyazaki, Tokyo, Japan) to [32P]-labeled oligonucleotides that included either wild-type sequence or the mutation-containing sequence was performed as described previously. The oligonucleotide sequences used are listed in Supplementary Table 4. Assay specificity was assessed by preincubation of nuclear lysates with 30- and 100-fold excess of unlabeled wild-type, mutant or consensus double-stranded oligonucleotide. Supershifts were performed using 2 μl of goat polyclonal serum to FOXA2 (sc-6554) or PDX1 (sc-14662) and control IgG (sc-2028) (all from Santa Cruz Biotechnology).

3C assays. Approximately 1 x 106 artificial pancreatic progenitors were fixed for 20 min at 4 °C in 4% paraformaldehyde, washed three times in PBS and lysed (10 mM Tris-HCl, pH 8, 10 mM NaCl, 0.3% IGEPAL CA-630 (Sigma-Aldrich, I8896) and 1× protease inhibitor cocktail (Complete, Roche)). Nuclei were digested with HindIII endonuclease (New England BioLabs). DNA was then ligated with T4 DNA ligase (Promega). Locus-specific primers were designed with Primer3 v. 0.4.0 (ref. 40) as described previously. The oligonucleotide sequences used are listed in Supplementary Table 4. Assay specificity was assessed by preincubation of nuclear lysates with 30- and 100-fold excess of unlabeled wild-type, mutant or consensus double-stranded oligonucleotide. Supershifts were performed using 2 μl of goat polyclonal serum to FOXA2 (sc-6554) or PDX1 (sc-14662) and control IgG (sc-2028) (all from Santa Cruz Biotechnology).

DNA was prepared with the PureYield Plasmid Maxiprep System (Promega). At day 10 of differentiation, artificial pancreatic progenitors were transfected in 24-well plates with 400 ng of pGL4.23-GW-PTF1A Enhancer vectors and 4 ng of Renilla normalizer control using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Gibco) according to the manufacturers’ instructions. Panc-1 (human pancreatic ductal; ATCC, CRL-1469), 266-6 (mouse pancreatic acinar; ATCC, CRL-2151), AR42 (rat pancreatic acinar; ATCC, CRL-1492) and HeLa (ATCC, CRM-CCL2) cells were transfected in 96-well plates using Lipofectamine 2000 in Opti-MEM at a density of 4 x 104 cells per well, according to the manufacturer’s instructions for this format. Luciferase activity was measured 48 h after transfection with the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity and then to the amount of pGL4.23[luc2/minP] vector backbone. Statistical significance was determined by comparing firefly/Renilla luciferase values for each mutant to those for the wild-type construct using a two-sided t test. All DNA preparations were transfected into cells triplicate.