Diabetes. Author manuscript; available in PMC 2016 September 01.

Published in final edited form as:
Diabetes. 2016 September ; 65(9): 2810–2815. doi:10.2337/db15-1666.

Isolated pancreatic aplasia due to a hypomorphic *PTF1A* mutation

Jayne A. L. Houghton[#1], **Galvin H. Swift**[#2], **Charles Shaw-Smith**[#1], **Sarah E. Flanagan**[1], **Elisa de Franco**[1], **Richard Caswell**[1], **Khalid Hussain**[3], **Sarar Mohamed**[4], **Majedah Abdulrasoul**[5], **Andrew T. Hattersley**[1], **Raymond J. MacDonald**[2], and **Sian Ellard**[1]

[1]Institute of Biomedical and Clinical Science, University of Exeter Medical School, Exeter, UK

[2]Department of Molecular Biology, University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd., Dallas, TX 75390-9148, USA

[3]Department of Endocrinology, Great Ormond Street Hospital, London, UK

[4]Department of Pediatrics, College of Medicine, King Saud University, Riyadh, Saudi Arabia

[5]Department of Pediatrics, Faculty of Medicine, Kuwait University, Kuwait

[#] These authors contributed equally to this work.

Abstract

Homozygous truncating mutations in the helix-loop-helix transcription factor *PTF1A* are a rare cause of pancreatic and cerebellar agenesis. The correlation of *Ptf1a* dosage with pancreatic phenotype in a mouse model suggested the possibility of finding hypomorphic *PTF1A* mutations in patients with pancreatic agenesis or neonatal diabetes but no cerebellar phenotype. Genome wide SNP typing in two siblings with neonatal diabetes from a consanguineous pedigree revealed a large shared homozygous region (31 Mb) spanning *PTF1A*. Sanger sequencing of *PTF1A* identified a novel missense mutation, p.P191T. Testing of 259 additional patients using a targeted next generation sequencing assay for 23 neonatal diabetes genes detected one additional proband and an affected sibling with the same homozygous mutation. All 4 cases were diagnosed with diabetes at birth and are insulin treated. Two of the 4 had exocrine pancreatic insufficiency requiring replacement but none of the affected individuals have neurodevelopmental delay. Transient transfection assays of the mutant protein demonstrated a 75% reduction in transactivation activity. This study shows that the functional severity of a homozygous mutation impacts on the severity of clinical features found in patients.

Corresponding authors: Prof. Andrew T. Hattersley, University of Exeter Medical School, Barrack Road, Exeter, EX2 5DW, UK, Tel: +44 (0) 1392 408260, FAX: +44 (0) 1392 408231, A.T.Hattersley@exeter.ac.uk. Prof Raymond MacDonald, UT Southwestern Medical Centre, 5323 Harry Hines Blvd, Dallas, TX 75390 USA, Tel: +1 214 648 1923, FAX: +1 214 648 1915, raymond.macdonald@utsouthwestern.edu.

Authors' contributions

SE, JALH, SEF, RC & EdF designed, performed and interpreted the genetic studies. AH, CS-S, KH, SM, & MA designed and performed the clinical studies. RJM and GHS designed, performed and interpreted the functional studies. JALH and GHS wrote the first draft of the paper with input from SE, ATH and RJM. All co-authors reviewed and commented on the draft manuscript and reviewed the submitted manuscript. S.E. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Keywords

neonatal diabetes; exocrine pancreatic insufficiency; cerebellar agenesis; PTF1A; hypomorphic mutation

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Introduction

Homozygous truncating mutations in the basic helix-loop-helix transcription factor *PTF1A* cause pancreatic agenesis and very severe neurodevelopmental problems including central hypoventilation and total cerebellar agenesis [1–3]. All 6 affected individuals from 4 families show a high degree of phenotypic concordance and none survived for more than 4 months.

A combined linkage, genome sequencing and epigenomic annotation strategy recently identified homozygous mutations in a novel enhancer located 25 kb downstream from the *PTF1A* gene [4]. Patients with biallelic mutations in this enhancer have isolated pancreatic agenesis without cerebellar involvement. Sparing of the cerebellum suggests that the enhancer is tissue-specific to the pancreas.

A mouse model relating *Ptf1a* dosage to pancreatic phenotype [5] resulted in pancreatic hypoplasia and glucose intolerance in a dosage-dependent manner. The pancreatic phenotype consisted of reduced pancreatic bud size, mis-specification of pancreatic progenitors, reduced branching morphogenesis of the exocrine pancreas and a reduction in the ratio of beta to non-beta cells in pancreatic islets. In this model system, *Ptf1a* RNA levels were correlated with the endocrine and exocrine pancreatic phenotype.

Biallelic mutations in a second transcription factor gene, *PDX1,* also result in non-syndromic pancreatic agenesis and have been identified in three unrelated cases [6–8]. Hypomorphic mutations result in neonatal diabetes in the absence of exocrine pancreatic insufficiency [9,10]. A hypomorphic mutation in the transcription factor gene *Pdx1* in mice has also been modelled [11] and shown to play a role in the transition from pancreatic progenitor to endocrine progenitor, with a reduction in the number of endocrine lineages.

To date, no *PTF1A* coding hypomorphic mutations have been identified. We now report four individuals from two separate sibships in each of whom the same novel *PTF1A* coding mutation, p.P191T, was identified. All four individuals were diagnosed with neonatal diabetes, but cerebellar pathology was absent. We performed functional studies on this mutation to investigate our hypothesis that p.P191T is a hypomorphic *PTF1A* mutation.

Methods

Genetics

Homozygosity mapping—Homozygosity mapping was carried out as described previously [12] in one patient with pancreatic agenesis of unknown aetiology and their sibling with neonatal diabetes. Pancreatic agenesis was defined as neonatal diabetes

requiring insulin treatment and exocrine pancreatic insufficiency requiring enzyme replacement therapy [13].

Next generation sequencing assay—A targeted next generation sequencing assay was used to sequence *PTF1A* and 22 other genes in which mutations have been reported to cause neonatal diabetes [14]. We sequenced DNA samples from 259 probands with neonatal diabetes diagnosed before 6 months and no known genetic aetiology. Nine of these patients had pancreatic agenesis. Mutations in *ABCC8, KCNJ11* and *INS* had previously been excluded by Sanger sequencing. We had also excluded *EIF2AK3* mutations in patients born to consanguineous parents.

Sanger sequencing—Mutations were confirmed by PCR/Sanger sequencing and tested in other relatives.

Functional work

Cell Transfections—The reporter plasmids Ela1p.luc, with a minimal promoter directing the luciferase gene of pGL3 basic, and 3Rbpjl.Ela1p.luc, with 3 tandem repeats of the proximal PTF1 binding site of the Rbpjl gene upstream of Ela1p.luc, have been described [15]. The p.P191T mutation was introduced into the human PTF1A coding sequence by site-directed mutagenesis as previously described [16]. DNA was introduced into the human embryonic kidney cell line HEK 293 (American Type Culture Collection CRL-1573) with FuGene6 (Roche, Indianapolis, INS, USA) according to the manufacturer's instructions. All transfections were normalized based on the β-galactosidase activity of a cotransfected reporter plasmid, pCMVbeta (Clontech, Mountain View, CA, USA).

Electrophoretic Mobility Shift Assays (EMSA)—The subunits of PTF1 were synthesized by *in vitro* transcription and translation (ivtt) using a TnT Reticulocyte Lysate System (Promega, Madison, WI, USA). The wild type and p.P191T mutant PTF1A plasmids described above were also used for *in vitro* protein synthesis. The plasmid bearing human RBPJ has previously been described [16]. The expression plasmid bearing a partial human E12/TCF3 cDNA in pCITE2a was a gift from Eric Olson, UT Southwestern, and was originally derived from E12R (20). The products of ivtt were quantified by [^{35}S]methionine incorporation and adjustment according to the number of methionine residues in each protein.

EMSAs were performed by the method of Sawada and Littman [17] with slight modifications. The double-stranded oligonucleotide probe encompassed the proximal PTF1 binding site of the mouse *Rbpjl* gene [15] and was 5'-end labeled with ^{32}P. The sequence of the top strand is GACACCTGCTGGGCAGATGTAGGCTTCCCACGG. ImageQuant software was used to analyze PhosphorImager scans of the EMSA gels (both, GE Healthcare, Life Sciences, Pittsburg, PA, USA).

Results

Homozygosity analysis of genome wide SNPs identified a homozygous region (31 Mb) on chromosome 10 which was shared between two affected siblings born to consanguineous

parents. Sanger sequencing of *PTF1A* identified a novel homozygous missense mutation, p.P191T (c.571C>A; p.Pro191Thr) in both siblings. Analysis of *PTF1A* by targeted next generation sequencing of 9 patients with pancreatic agenesis identified one additional proband with the p.P191T mutation. This patient's affected sibling was also homozygous for p.P191T. No additional *PTF1A* mutations were identified in a further 250 patients with neonatal diabetes.

P191T mutation characteristics

The p.P191 residue is located in the highly conserved helix loop helix domain which is critical for dimerisation and DNA binding (Figure 1). The p.P191T missense variant is not present in any public variant databases (1000 Genomes, NHLBI Exome Sequencing Project Exome Variant Server (EVS) or the ExAC browser). *In silico* analysis by SIFT and PolyPhen predicts the variant to be pathogenic. All four affected patients were homozygous for the p.P191T mutation and their parents were heterozygous carriers. The patients are from Saudi Arabia and Kuwait, raising the possibility of a founder mutation. Analysis of microsatellite markers flanking *PTF1A* showed a shared haplotype of 8 Mb (data not shown), consistent with a founder mutation segregating in the two families.

Clinical characteristics of patients with homozygous P191T mutation

The male proband in family I, (I-1), was the first child born to first cousins of Arabic descent with a birth weight of 1.98kg at 38 weeks' gestation. He was noted to be hyperglycemic (350 mg/dl) on the first day of life while on a glucose infusion which was discontinued and an insulin infusion commenced for 20 hours. On Day 6, when on no treatment, his plasma glucose remained high (240 mg/dl) with low insulin of 0.1 MU/L (N 1.4-14) and C peptide of 0.1 ng/ml (N 0.9-4.3) confirming a diagnosis of neonatal diabetes. Insulin was recommend at 2 months of age as most glucose values were 200-300 mg/dl and his HbA1c was 7%. This has continued since then with Hba1c now 8.1%. The baby continued to have poor weight gain despite recommencing insulin and steatorrhoea was observed suggesting pancreatic insufficiency which was confirmed by elevated fecal fat, elevated stool chymotrypsin and low serum trypsinogen (Table 1). Exocrine pancreatic replacement therapy was initiated at 3 months of age. Other clinical features were a small patent ductus arteriosus, with a small atrial septal defect which were detected by echo after a cardiac murmur was noted; these did not require any intervention.

His younger sister (I-2) was born at 37 weeks' gestation, birth weight 2.00 kg. Diabetes was diagnosed on the first day of life with a glucose of 300mg/dl and treated with insulin for 4 days. Insulin was commenced at four months due to consistently high blood glucose values (> 200mg/dl) and HbA1c of 7% and has continued since then with HbA1c values 7-8%. Exocrine pancreatic supplementation has not been required and biochemical parameters (fecal fat, stool chymotrypsin and serum trypsinogen) are within normal limits, albeit at the lower limit of the normal range (see Table 1).

Ultrasound scanning of the abdomen of the two siblings in family I was performed in the first week of life and showed normal liver and contracted gallbladder, but failed to identify the pancreas. Parents declined further imaging of the abdomen.

Both siblings in family I underwent detailed neurodevelopmental assessment by a neurodevelopmental pediatrician at the age of 12 years in the older and at 9 years in the younger sibling. Evaluation revealed normal neurological examination apart from searching eye movements with horizontal nystagmus in patient 1. There are no other clinical abnormalities referable to cerebellar function. Parents declined MRI brain imaging. The rest of the clinical neurodevelopmental assessment was age-appropriate including vision, hearing, expressive and receptive language, gross motor and fine motor skills, social skills, and school performance. Their clinical neurodevelopmental assessment is similar to their unaffected siblings. However, formal neuropsychological testing was not performed.

The male proband in family II (II-1) was the first child born to consanguineous parents of Arabic descent. Birth weight was 1.275 kg at 34 weeks' gestation. Neonatal diabetes was diagnosed on the first day of life and treated with insulin. There was severe failure to thrive gaining only 250g in the first 12 weeks of life but biochemical tests for pancreatic malabsorption were not performed. The patient died of necrotising enterocolitis and overwhelming sepsis at the age of 12 weeks. No further clinical details are available.

His younger sibling is a female child born at 36 weeks' gestation with birth weight 1.40 kg. Neonatal diabetes was diagnosed at the age of 8 days and treated with insulin. She failed to thrive and fecal elastase was undetectable, confirming a diagnosis of exocrine pancreatic insufficiency. Exocrine replacement therapy was initiated at 10 weeks' of age. Neurocognitive development and neurological examination are normal at the age of 2 years. Brain and cerebellar MRI scan was normal aged 3 months.

Functional work

Transfection of the p.P191T mutation into HEK293 cells resulted in a 75% reduction in transcriptional activity compared to the wild type protein (Figure 2). A range in the amount of expression plasmids was tested to ensure that the activities of the wild type plasmids were proportional to the plasmid quantity and, thus, not saturated in the assay. The mean activity of the mutant PTF1A at three different concentrations within the linear range was 25 +/- 4.9% of the activity of the wild type protein ($p < 0.001$) (Figure 2).

To determine the DNA binding characteristics of human PTF1A -P191T relative to wild type EMSAs were performed with a *Rbpjl* proximal PTF1 binding site as the radiolabeled probe (Figure 3). For the PTF1A:E12 dimer, the binding of the p.P191T mutant was 34% +/-7% of wild type (n = 3) whilst for the PTF1A:E12:Rbpj trimer, the binding of the p.P191T mutant was 88% +/-19% of wild type (n = 4).

Discussion

We report a novel hypomorphic homozygous *PTF1A* mutation in 4 affected individuals from two families who are likely to have inherited the mutation from a common distant ancestor. The clinical phenotype is of neonatal diabetes with reduced exocrine function and normal neurological function and appearance. This clinical phenotype is distinct from the previously reported syndrome caused by homozygous *PTF1A* truncating mutations where all 6 patients (from 4 families) died in the first 4 months of life [1–3] of severe neurological complications

as well as having pancreatic and cerebellar agenesis. *In vitro* functional studies are consistent with the p.P191T missense mutation being a hypomorphic mutation.

The main clinical feature of homozygosity for the p.P191T missense mutation is pancreatic aplasia/hypoplasia. All 4 patients had neonatal diabetes and 2 of the 4 have clinical and biochemical evidence of clinically significant exocrine dysfunction of the pancreas requiring replacement therapy. Another patient had clinical features suggestive of exocrine insufficiency but died of sepsis at 12 weeks and the fourth patient had diabetes requiring insulin treatment but no clinical features of exocrine failure, albeit with pancreatic enzyme levels close to the lower limit of the normal range.

All four patients had normal neurological development and function. This is in contrast to the patients with homozygous null *PTF1A* mutations who had very severe neurological developmental problems, central hypoventilation and total cerebellar agenesis. One patient had some disrupted eye movements but the phenotype was not consistent with significant cerebellar dysfunction. A normal cerebellum was seen in the patient who had brain imaging.

The moderate phenotypic variability seen in these 4 patients is also seen in patients who have other pancreatic transcription factor mutations affecting the pancreatic stem cell. Different phenotypes due to the same mutation are seen in patients with mutations in *GATA6* [18], and in *HNF1B* [19]. Phenotypic variability is also seen in patients with mutations in the homeodomain transcription factor *PDX1*: biallelic mutations in this gene have been reported in patients with pancreatic agenesis [6–8]; neonatal diabetes with biochemical but not clinical evidence of exocrine insufficiency [9,10], and neonatal diabetes with normal exocrine function, both clinically and biochemically [10].

The p.P191T missense mutation is located in the highly conserved helix loop helix domain of *PTF1A*, a region which is critical for dimerisation with a common bHLH E-protein such as E12/E47 and DNA binding. Proline 191 terminates helix 1, and substitution of threonine at that position could favor the extension of the first α-helix, almost a full turn and thereby shorten the loop region, mis-position the second α-helix, and make binding to the E-protein partner less favourable. Indeed, *in vitro* functional studies demonstrated an effect on DNA binding and transactivation activity was reduced by 75% compared to the wild-type protein. This residual activity is consistent with p.P191T being a hypomorphic mutation.

Reduced *Ptf1a* dosage in a mouse model [5] resulted in pancreatic hypoplasia and glucose intolerance in a dosage-dependent manner. In hypomorphic mutant mice, pancreatic bud size was small and substantial proportions of pancreatic progenitors were mis-specified to the common bile duct and duodenal cells. Exocrine pancreatic branching morphogenesis was reduced, and there was a reduction in the ratio of beta cells to non-beta cells in pancreatic islets. In this model system, *Ptf1a* RNA levels could be correlated with the endocrine and exocrine pancreatic phenotype. We have clearly shown a similar correlation between mutation severity and phenotype in humans.

We conclude that hypomorphic *PTF1A* missense mutations can cause isolated pancreatic agenesis or neonatal diabetes. Hypomorphic mutations in this gene should be considered in patients presenting with diabetes mellitus in the neonatal period.

Acknowledgements

The authors thank the families for participating in this study. We are grateful to Annet Damhuis and Anna-Maria Bussell and the Research Center, College of Medicine, King Saud University for their technical assistance and to Ward Coats for helpful discussions regarding PTF1A structure. ATH and SE are the recipients of a Wellcome Trust Senior Investigator award and this funded the genetic/clinical part of this study (WT 098395). ATH is employed as a core member of staff within the NIHR funded Exeter Clinical Research Facility and is a NIHR senior investigator. GHS and RJM were supported by NIH grant R01-DK061220.

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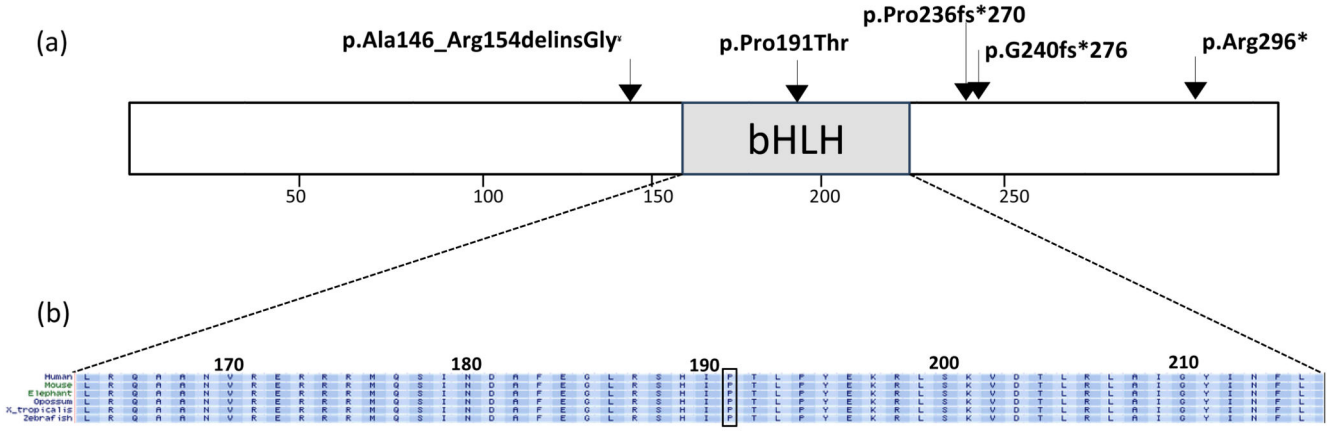

Figure 1.

(a) Schematic representation of the PTF1A protein and location of the mutations identified in all previously reported cases [1–3], and the present case. The p.P191T is the only missense mutation identified to date. It is located at a conserved residue within the bHLH domain (highlighted). (b) Amino acid conservation in the bHLH domain. The location of the p.P191T mutation found in the 4 patients us shown. ¥ The p.Ala146ArgdelinsGly mutation was previously reported as c.437_460del, p.Ala146_Arg154delfsX115 (3).

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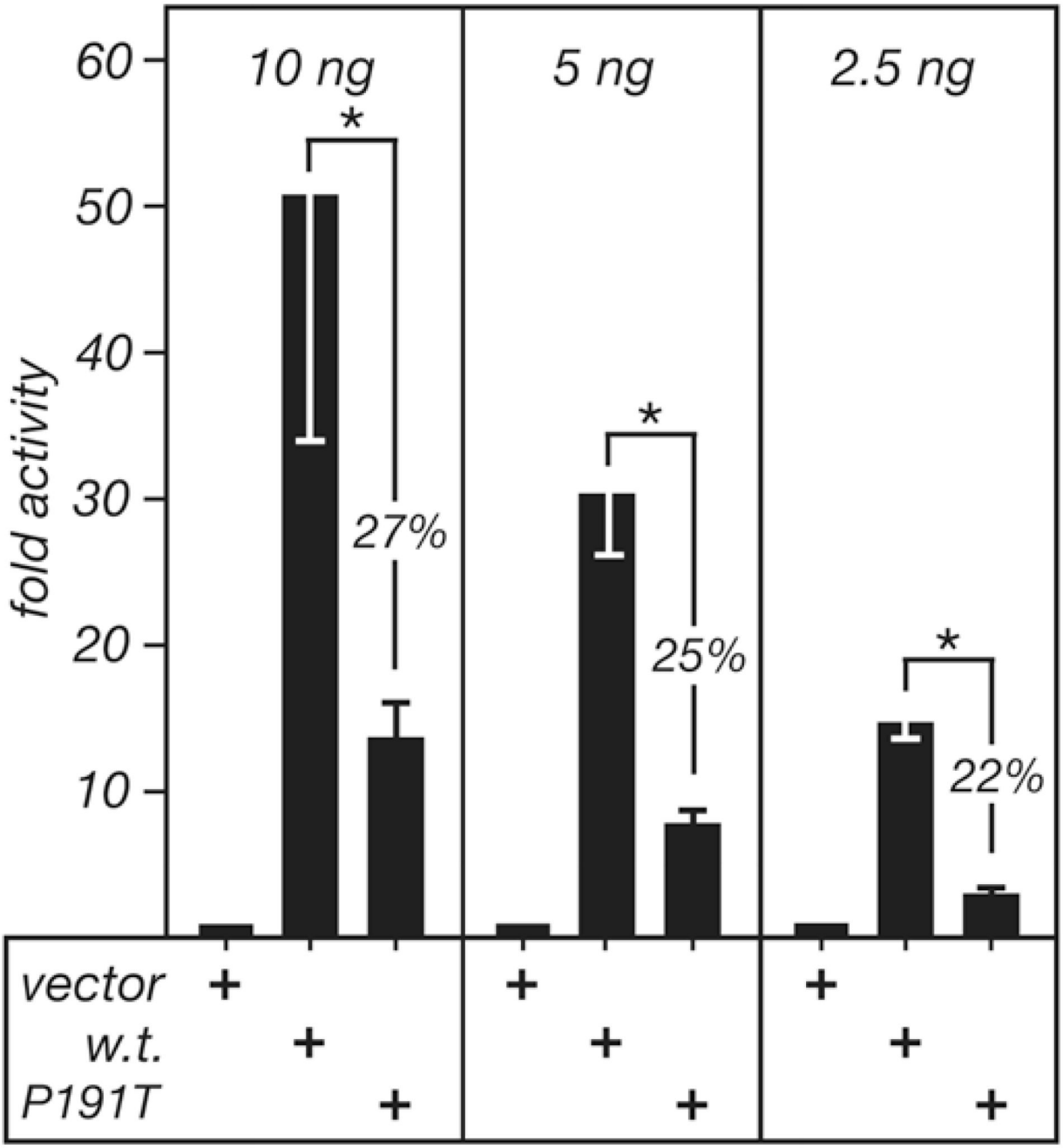

Figure 2. The activity of human PTF1A-P191T in transfected cells.

The reporter plasmid 3Rbpjl.Ela1p.luc was cotransfected with expression plasmids for wild type PTF1A, PTF1A-P191T, or an insertless vector into human embryonic kidney (HEK) 293 cells. Three different amounts of each expression plasmid were used to determine whether response was proportional to plasmid quantity and, therefore, that the proteins produced were not in excess. Four independent transfections were analyzed for 10 and 5ng, and two for 2.5ng. Error bars are standard deviations. The asterisks indicate p values <0.05 for each pairwise comparison.

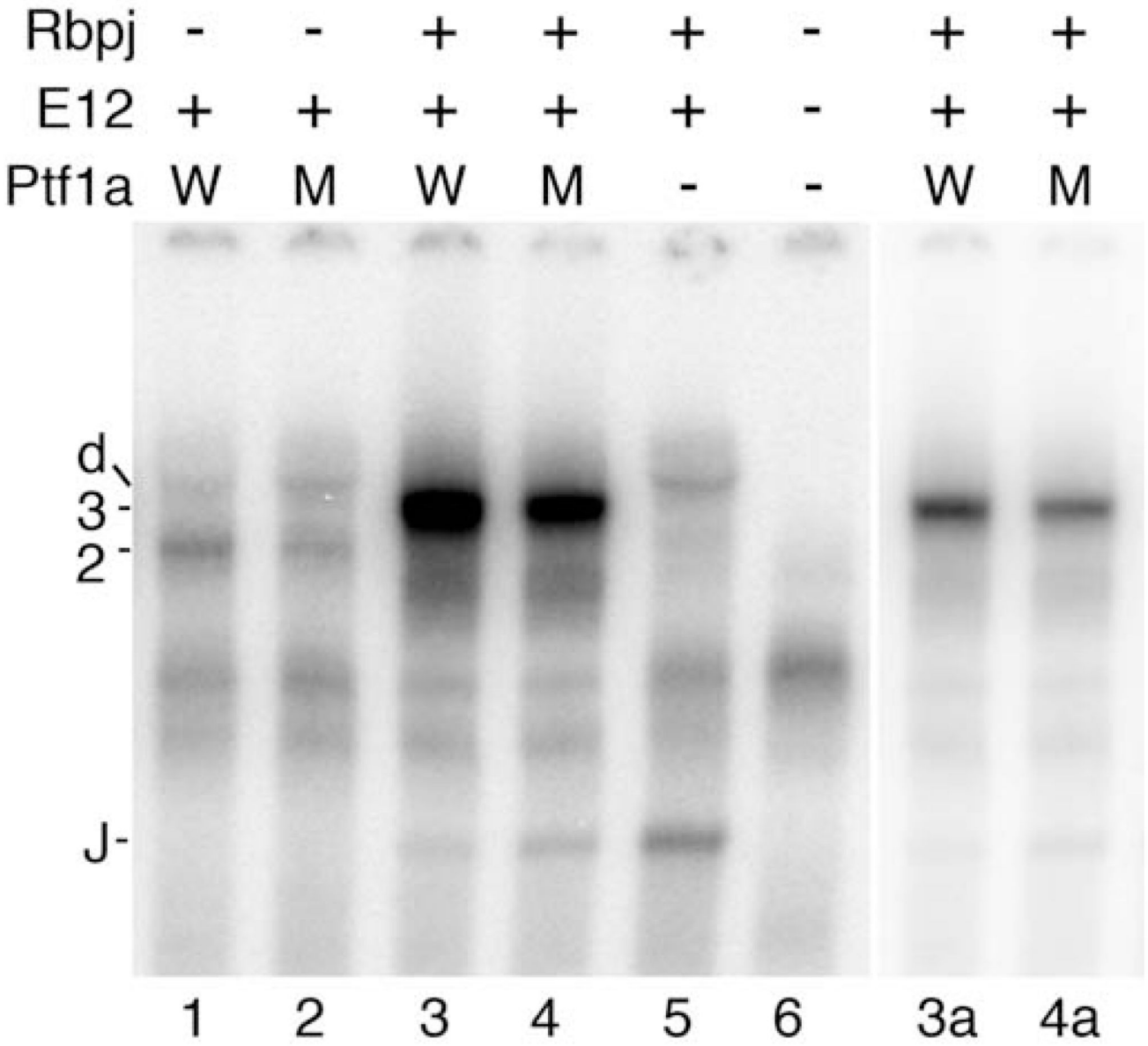

Figure 3. The DNA binding of wild type PTF1A and PTF1A-P191T as part of the PTF1A:E12 dimer or PTF1A:E12:Rbpj trimer in EMSA.

The relative abilities of wild type and p.P191T mutant Ptf1a to form complexes with E12 and Rbpj on the PTF1 binding site of the Rbpjl gene was tested *in vitro*. W, Ptf1a wild type; M, p.P191T mutant; 2, Ptf1a:E12 dimer; 3, Ptf1a:E12:Rbpj trimer; d, E12 homodimer; J, Rbpj monomer. Lanes 3a and 4a show lanes 3 and 4 with an expanded gray scale such that the PTF1 trimeric complex bands are not saturated. Ptf1a alone does not bind DNA.

Table 1

Pancreatic clinical characteristics of patients studied.

Patient	I-1	I-2	II-1	II-2
Present age	12 years	9 years	Deceased at 12 weeks due to sepsis	2 years
Sex	Male	Female	Male	Female
Country of origin	Saudi Arabia	Saudi Arabia	Kuwait	Kuwait
Birth weight Percentile	1980 g 0.4	2000 g 2	1275 g 0.1	1400 g <0.1
Gestational age	38 weeks	37 weeks	34 weeks	36 weeks
Age at diabetes diagnosis/ Age of permanent Insulin therapy	1 Day /2 months	1 day/4 months	1 day/1 day	8 days/ 8 days
Diabetes treatment	Insulin	Insulin	Insulin	Insulin
Exocrine pancreatic insufficiency requiring replacement?	Yes	No	Yes	Yes
Clinical basis of exocrine insufficiency	Steatorrhoea, failure to thrive	No symptoms of exocrine insufficiency	Failure to thrive	Steatorrhoea, failure to thrive
Biochemical basis of exocrine insufficiency	Fecal fat: 3 g/ 24hr stool Stool chymotrypsin: 1 U/g (4-10U/g) Serum trypsinogen: 4 μg/l (15-25 μg/l)	Fecal fat: 0.5 g/24hr stool Stool chymotrypsin: 5 U/g (4-10U/g) Serum trypsinogen: 18 μg/l (15-25 μg/l)		Fecal elastase: undetectable (>200 μg elastase/g stool)
Exocrine pancreatic replacement regimen	10 000 U/day of lipase	None		15 000 U/day of lipase
Age at initiation of exocrine pancreatic replacement	12 weeks	N/A	N/A	10 weeks
Clinical neurocognitive function	Normal neurocognitive development with some disruption of eye movements on tracking.	Normal neurocognitive development		Normal neurocognitive development
Brain imaging	Not performed	Not performed	Not performed	Normal (MRI imaging)