Mutations in *NEUROD1* are associated with the development of type 2 diabetes mellitus

Maciej T. Malecki[1,3*], Ulupi S. Jhala[1*], Anthony Antonellis[1], Liz Fields[1], Alessandro Doria[1], Tihamer Orban[1], Mohammed Saad[2], James H. Warram[1], Marc Montminy[1] & Andrzej S. Krolewski[1]

These authors contributed equally to this work.

The helix-loop-helix (HLH) protein NEUROD1 (also known as BETA2) functions as a regulatory switch for endocrine pancreatic development. In mice homozygous for a targeted disruption of *Neurod*, pancreatic islet morphogenesis is abnormal and overt diabetes develops due in part to inadequate expression of the insulin gene[1] (*Ins2*). NEUROD1, following its heterodimerization with the ubiquitous HLH protein E47, regulates insulin gene (*INS*) expression by binding to a critical E-box motif on the *INS* promoter[2]. Here we describe two mutations in *NEUROD1*, which are associated with the development of type 2 diabetes in the heterozygous state. The first, a missense mutation at Arg 111 in the DNA-binding domain, abolishes E-box binding activity of NEUROD1. The second mutation gives rise to a truncated polypeptide lacking the carboxy-terminal *trans*-activation domain, a region that associates with the co-activators CBP and p300 (refs 3,4). The clinical profile of patients with the truncated NEUROD1 polypeptide is more severe than that of patients with the Arg 111 mutation. Our findings suggest that deficient binding of NEUROD1 or binding of a transcriptionally inactive NEUROD1 polypeptide to target promoters in pancreatic islets leads to the development of type 2 diabetes in humans.

NEUROD1 contains two exons and has been mapped to chromosome 2q (refs 5,6). We did not examine exon 1 because it is not translated[7]. Exon 2 encodes a protein with several distinct domains (Fig. 1*a*). We screened exon 2 and the flanking intron sequences for DNA sequence differences by direct sequencing of DNA samples from 94 individuals with type 2 diabetes. Each was the index case through which we ascertained 94 large families for the presence of diabetes segregating as an autosomal dominant disorder[8,9].

We examined exon 2 in all index cases and found four variants of the published sequence. The first was a single base-pair substitution, G→A, in codon 45 that results in an Ala→Thr substitution in the amino terminus of NEUROD1. The frequency of the threonine variant was similar in 94 index cases and in 96 unrelated non-diabetic individuals (32.9% and 35.9%, respectively). Similarly, this polymorphism was not associated with

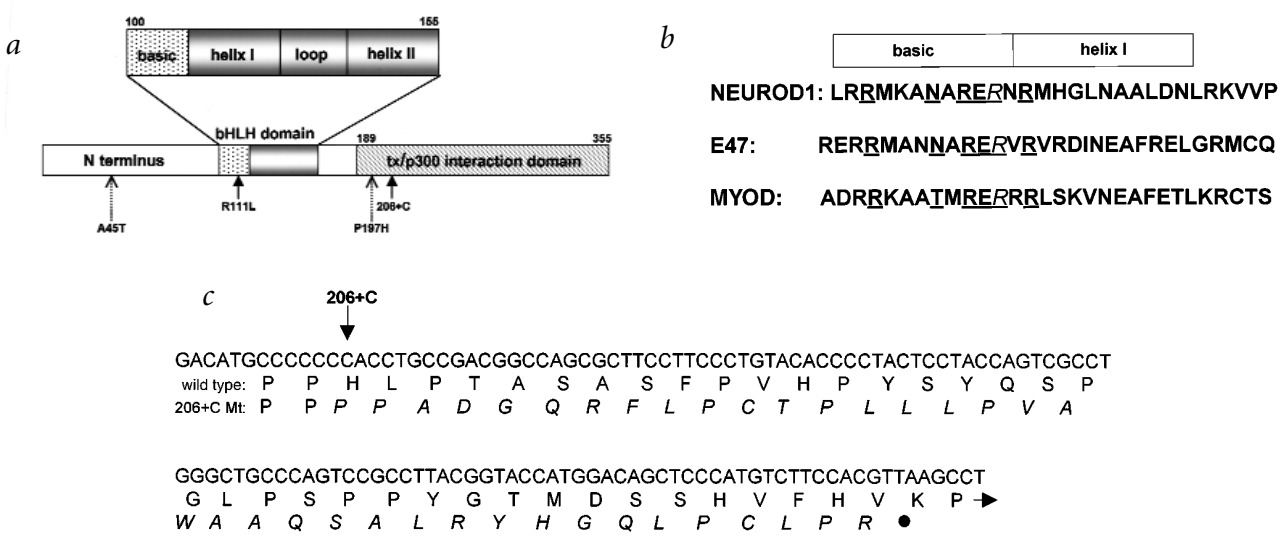
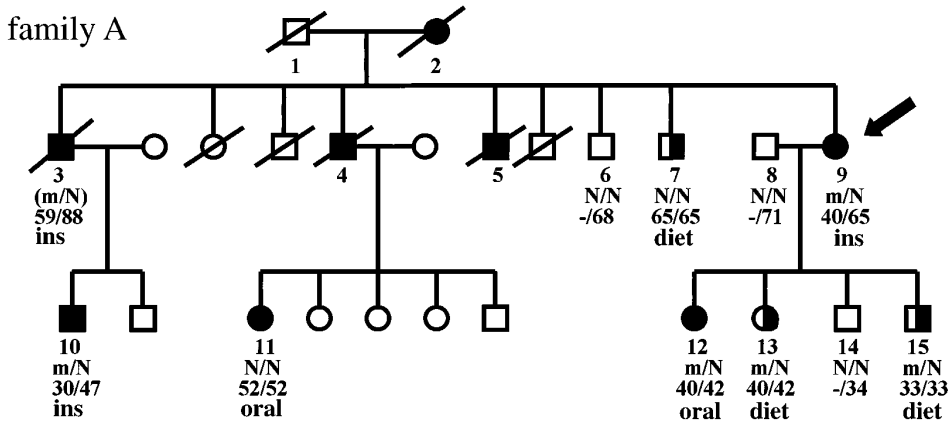


Fig. 1 Sequence differences found in *NEUROD1*. ***a***, Schematic organization of NEUROD1 and its domains. Numbers refer to the amino acids bordering the domains. The details of the HLH domain are shown at the top. Filled arrows indicate mutations and the dotted arrows indicate the amino acid variants identified in *NEUROD1*. The borders were determined based on mammalian homology using published data[2]. 'tx/p300' indicates the transactivation domain as well as the p300-interacting region of NEUROD1 (refs 3,4). ***b***, Alignment of the first 30 aa of the bHLH domain of NEUROD1 with other members of the basic HLH (bHLH) family. Residues responsible for DNA contact are underlined. The Arg 111 residue of NEUROD1 and the corresponding residues of MYOD and E47 are shown[16–18] (italics). ***c***, A fragment of *NEUROD1* sequence is shown, with the 206+C insertion indicated by an arrow. Bottom, the corresponding amino acid sequences of wild type and the 206+C mutation. This mutation resulted in the generation of a truncated protein lacking the C terminus.

[1]*Research Division, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts, USA.* [2]*Division of Endocrinology, UCLA School of Medicine, Los Angeles, California, USA.* [3]*Present address: Department of Metabolic Diseases, Jagellonian University School of Medicine, Cracow, Poland. Correspondence should be addressed to A.S.K. (e-mail: andrzej.krolewski@joslin.harvard.edu).*

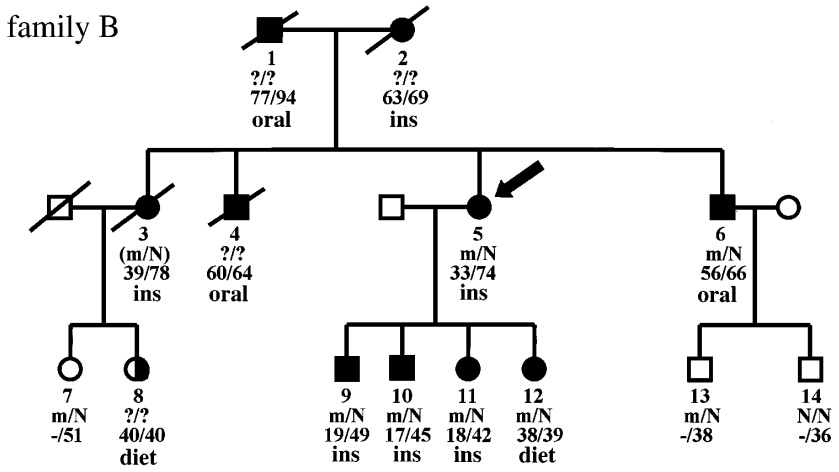


ID	Clinical status	% IBW	Glucose levels (mg %)		C peptide (ng/ml)	Insulin levels (μU/ml)	
			0′	120′		0′	120′
09	DM	136	265		0.9		
10	DM	126	125		1.2		
12	DM	144	155	277		18	52
13	IGT	141	116	163		15	78
15	IGT	143	102	165		18	49
67 type 2 cases		143±36	144±56	245±102		17±15	54±44

Fig. 2 Pedigree, genotypes and clinical characteristics of family A. Top, segregation of the R111L mutation. FIlled, half-filled and open symbols represent subjects with type 2 diabetes, impaired glucose tolerance and normal glucose tolerance, respectively. The numbers under the symbols are the identification numbers. Below this is the genotype at codon 111: N, normal allele (Arg); m, mutant allele (Leu); the genotype in parenthesis has been inferred. Below the genotype is the age at diabetes diagnosis for affected members and age at examination or age at death for deceased members, followed by the treatment for diabetes. An arrow indicates the index case. Parametric lod score for this pedigree was 0.65. Bottom, other clinical characteristics for carriers of the mutation. For comparison, mean and standard deviation for the same characteristics in a group of 67 individuals with type 2 diabetes are shown[9]. Individuals treated with insulin did not have the oral glucose tolerance test performed.

type 2 diabetes in Japanese and French populations[7,10]. The second polymorphism was a single base pair substitution, C→A, in codon 197 that results in a Pro→His substitution in the C terminus of NEUROD1 (Fig. 1*a*). This variant was equally frequent in diabetic and non-diabetic subjects (3.6% and 3.1%, respectively). The two remaining sequence differences were found in two index cases and were not present in controls.

The index case from family A had a G→T substitution in codon 111, causing an Arg→Leu change (designated R111L). This mutation is located in the proximal basic portion of the basic HLH domain, which is responsible for DNA binding (Fig. 1*a*). This Arg111 residue in the DNA-binding domain of NEUROD1 has been evolutionarily conserved from *Drosophila melanogaster* to mammals and is a characteristic shared with other members of the HLH family[1] (Fig. 1*b*). The index case from family B had an insertion of a cytosine residue in a polyC tract in codon 206 in exon 2 (designated 206+C), resulting in a frameshift and synthesis of a nonsense peptide from amino acid 205 to 242, followed by a premature stop codon (Fig. 1*c*). Thus, the 206+C mutation caused the loss of the C-terminal third of the protein, which harbours the transactivation domain[8] and interacts with the cellular coactivator p300 (refs 3,4).



ID	Clinical status	% IBW	Glucose levels (mg %)		C peptide (ng/ml)	Insulin levels (μU/ml)	
			0′	120′		0′	120′
05	DM	104	189		<0.2		
09	DM	111	186		0.5		
10	DM	134	174		<0.2		
11	DM	113	155		0.6		
06	DM	110	143	302		14	16
12	DM	113	101	201		5	14
13	N	132	74	71		6	7
07	N	102	96	74		11	38
29 MODY3 cases		113±21	116±37	259±97		7±5	11±10

Fig. 3 Pedigree, genotypes and clinical characteristics of family B. Top, segregation of the 206+C mutation. Filled, half-filled and open symbols represent subjects with type 2 diabetes, impaired glucose tolerance and normal glucose tolerance, respectively. The numbers under the symbols are the identification numbers. Below this is the genotype at codon 206: N, normal allele; m, mutant allele (206+C, resulting in truncated protein); the genotype in parenthesis has been inferred. Below the genotype is the age at diabetes diagnosis for affected members and age at examination or age at death for deceased members, followed by the treatment for diabetes. An arrow indicates the index case. Parametric lod score for this pedigree was 0.90. Bottom, other clinical characteristics for carriers of the mutation. At the time of examination, two carriers (5 and 10) did not secrete endogenous insulin, but they had been treated with diet or oral agents for many years after the diagnosis of diabetes. For comparison, means values and standard deviations for the same characteristics in a group of 29 MODY3 individuals are shown[9]. Individuals treated with insulin did not have the oral glucose tolerance test performed.

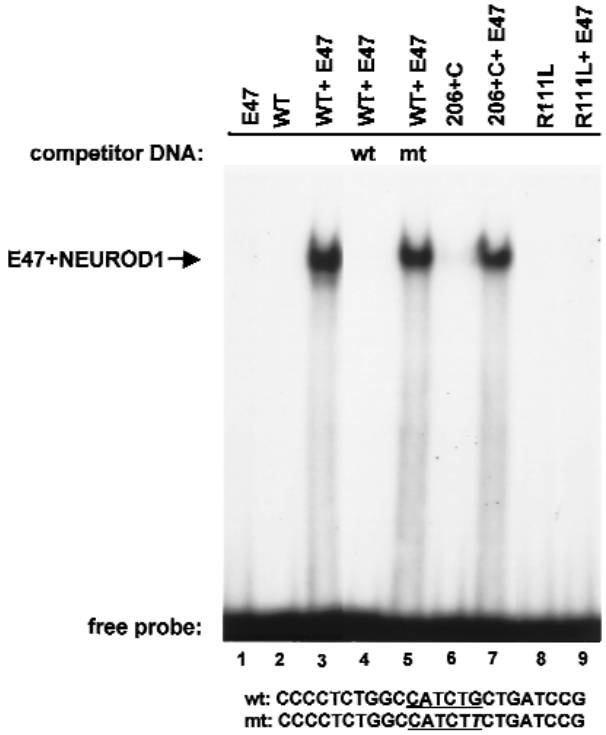


Fig. 4 DNA-binding properties of NEUROD1 mutants. Bacterially expressed wild-type (WT) or mutant (206+C or R111L) GST-NEUROD1 proteins were assessed for their ability to bind a ^{32}P-labelled insulin RIPE3 probe[2] by gel mobility shift assay in the absence and presence of E47. E47 and NEUROD1 individually display poor DNA-binding capacity (lanes 1 and 2), but the two proteins together bound avidly to the insulin E-box probe (lane 3). Specificity of binding was ascertained by competition of the NEUROD1-E47 complex with 100-fold molar excess of unlabelled wild-type or mutant RIPE3 double-stranded DNA (lanes 4 and 5). Sequences of wild-type and mutant probes are shown with the consensus E-box (underlined) and the mutation indicated (italics). The NEUROD1-206+C (lanes 6 and 7) mutant displayed binding characteristics similar to those observed for WT-NEUROD1 (lanes 2 and 3), but R111L was unable to bind DNA even in the presence of E47 (lanes 8 and 9). Experiments were repeated three times and showed the same pattern.

We determined the presence of these mutations in other members of the two families by direct sequencing. In family A, of six carriers of the R111L mutation (five observed and one inferred), four had previously diagnosed diabetes and two had impaired glucose tolerance diagnosed at the time of examination (Fig. 2). The average age of these 4 carriers at the time of diagnosis was 40 (range 30–59) years. In addition, a non-carrier aged 52 had diabetes treated by oral medication, and a non-carrier aged 65 had impaired glucose tolerance. These two individuals are most likely phenocopies, a phenomenon manifested in MODY pedigrees[11,12]. All carriers were obese (average per cent ideal body weight was 138%), and they had relatively high fasting serum levels of insulin (or C peptide in those treated with insulin) and high levels two hours after oral glucose. These individuals resemble 'typical' patients with onset of type 2 diabetes in middle age. Four non-carriers of this mutation were equally obese (average per cent ideal body weight was 136%). These data indicate that obesity was not associated with the R111L mutation.

In family B, the nine carriers of the 206+C mutation (eight examined and one inferred) included seven previously diagnosed diabetic and two non-diabetic individuals, apparently non-penetrants (Fig. 3). The average age of these 7 carriers at the time of diagnosis was 31 (range 17–56) years, and at the time of examination their diabetes was treated with diet or oral agents or insulin. In contrast to family A, individuals in family B were not obese (average per cent ideal body weight was 115%). All had low serum insulin levels, and two who treated their diabetes with insulin had undetectable serum C peptide, indicating the absence of endogenous insulin secretion. These characteristics resemble the MODY3 phenotype of carriers of *TCF1* (also known as *HNF1*α) mutations[9,13].

No carriers in either family had elevated levels of IA2 or GAD antibodies (markers of immunological destruction of β-cells) or physician-diagnosed disorders of the central nervous system or gastrointestinal tract, two other tissues where *NEUROD1* is expressed[1,14,15].

Comparison of the sequence of NEUROD1 with other basic HLH proteins revealed that Arg 111 in NEUROD1 is an invariant residue critical for DNA recognition[16,17]. Indeed, mutagenesis at this site in E47 protein disrupts DNA binding, suggesting that the R111L substitution in NEUROD1 may have similar effects[18]. In gel mobility shift assays, wild-type NEUROD1 polypeptide and the 206+C truncated variant bound with high affinity to the E-box in the insulin RIPE3 element in conjunction with E47. In contrast, the R111L polypeptide variant was unable to bind to the RIPE3 E-box, even in combination with E47 (Fig. 4).

To evaluate the effects of *NEUROD1* mutations on insulin gene expression, we performed transient assays using the rat *Ins2* promoter, which contains a single E-box for NEUROD1/E47 heterodimer[2]. Following transfection into Min6 insulinoma cells, a wild-type NEUROD1 expression vector induced insulin promoter activity sevenfold. Consistent with previous findings that NEUROD1 interacts functionally with the co-activator p300, overexpression of p300 further potentiated insulin reporter activity 2.5-fold. By contrast, both mutant NEUROD1 polypeptides, 206+C and R111L, were far less active on the *Ins2* promoter (2-fold and 1.5-fold activation, respectively), despite being expressed at levels comparable to wild-type NEUROD1 in transfected cells (Fig. 5*a*,*c*). Potentiation of insulin reporter activity via p300 also decreased, albeit more severely in cells transfected with R111L than in those with 206+C.

To rule out regulatory contributions from the E47 heterodimerization partner of NEUROD1, and to evaluate whether the R111L substitution disrupts NEUROD1 activity solely at the level of DNA recognition, we performed transient assays on Gal-NEUROD1 chimaeras with the Gal4 DNA-binding domain fused in-frame to the N terminus of NEUROD1. Wild-type Gal-NEUROD1 induced CAT reporter activity sixfold, and in the presence of p300, CAT activity was induced 12-fold (Fig. 5*d*). The activity of the mutant Gal-NEUROD1-R111L was comparable with that of wild-type Gal-NEUROD1, further supporting the fact that replacement of arginine with leucine attenuates target gene expression by disruption of DNA recognition. The truncated mutant construct, Gal-NEUROD1-206+C, was less active in stimulating the Gal4-CAT reporter, demonstrating the importance of the C-terminal domain of NEUROD1 for target gene activation.

NEUROD1 stimulates target gene activation by binding to the C terminus of the co-activator CBP and its paralogue p300 (refs 3,4,15). In GST pull-down assays, wild-type and mutant R111L NEUROD1 bound comparably to the C terminus of p300 (aa 1,572–2,370). Accordingly, overexpression of full-length p300 potentiated target gene activation via wild-type Gal-NEUROD1 and Gal-NEUROD1-R111L polypeptides. In contrast, the NEUROD1 206+C mutant was unable to associate with p300 in GST pull-down assays, and overexpression of p300 had little effect on Gal-NEUROD1-206+C activity (Fig. 6).

We demonstrate that the development of type 2 diabetes in carriers of the R111L and 206+C mutations is due to a disruption of NEUROD1 activity via two distinct mechanisms: (i)

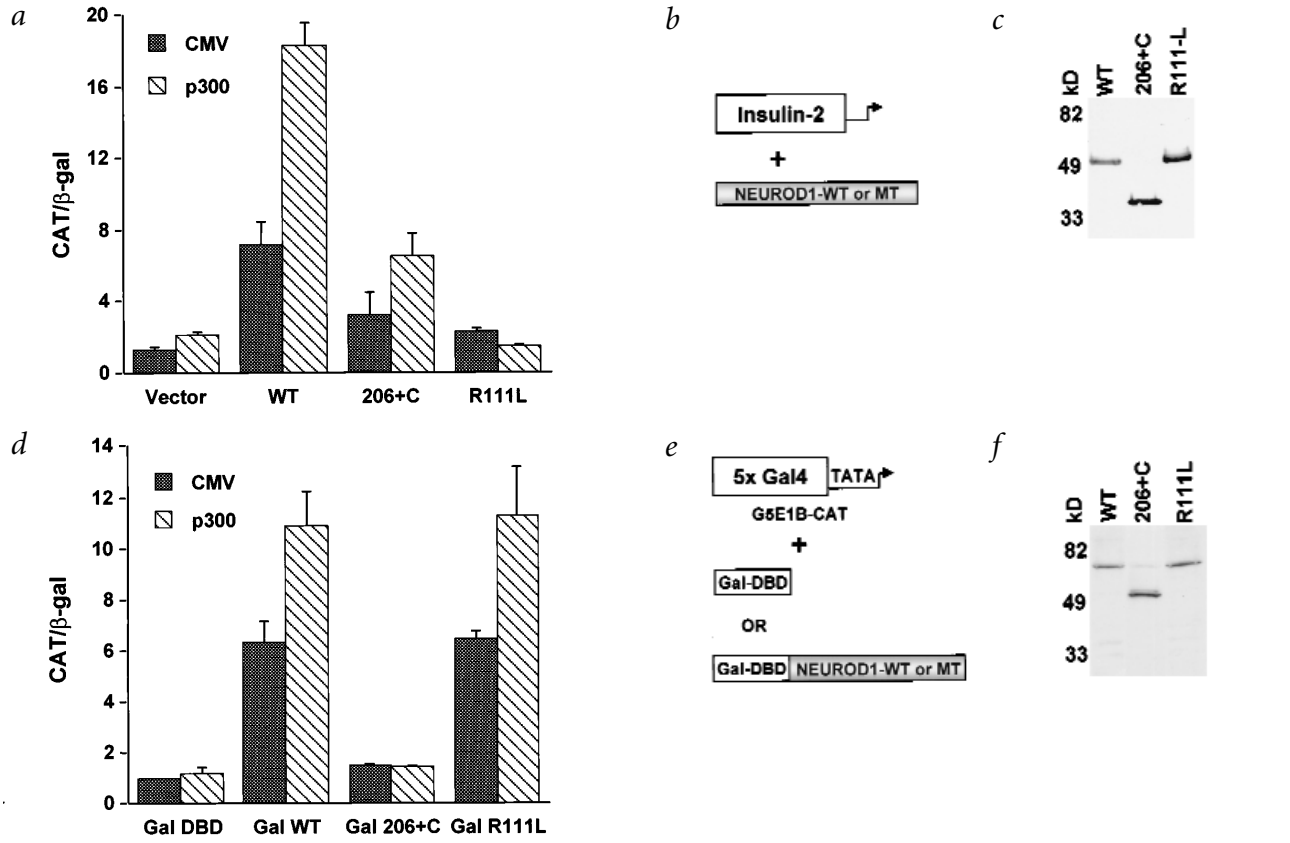


Fig. 5 Attenuation of transactivation by the NEUROD1 R111L and 206+C mutants. ***a***, Effect of *NEUROD1* mutations on rat *Ins2* activity. Min6 insulinoma cells transfected with rat *Ins2* CAT reporter gene and vectors encoding Flag-tagged NEUROD1 (wild type or mutant) in the presence of control vector (CMV) or p300 were assessed for CAT reporter activity. Dark shaded bars represent *Ins2* activity in the presence of empty vector (pCDNA3) or NEUROD1 wild-type and mutant proteins in the absence of p300, and hatched bars represent activity in presence of p300. ***b***, Schematic of constructs used in the transfection experiments. ***c***, Western-blot analysis for comparable expression of Flag-tagged NEUROD1 wild-type and mutant proteins using anti-Flag antibodies is shown. ***d***, Effect of *NEUROD1* mutations on G5-E1B-CAT reporter activity. HepG2 cells transfected with G5-E1B-CAT reporter and vectors encoding either Gal protein or Gal-NEUROD1 (wild type or mutant) fusion proteins, in the presence of control (CMV) vector or p300, were assessed for CAT reporter activity. Dark shaded bars represent expression of the Gal-DNA binding domain (GalDBD) as a control, or expression of Gal-NEUROD1 (wild type or mutant 206+C or R111L) fusion constructs in the presence of empty vector (CMV), and hatched bars indicate activity in presence of the fusion constructs with p300. Note the virtual lack of transactivation by NEUROD1 206+C irrespective of the presence of p300. ***e***, A schematic representation of the constructs used in the transfection assay is shown. ***f***, Western-blot analysis of Gal-NEUROD1 proteins using anti-Gal antibodies. Gal-NEUROD1 fusion proteins were tested for comparable expression in nuclear extracts of transfected HepG2 cells using mouse monoclonal antibody against Gal protein expressed N terminal to the NEUROD1 proteins. The results in (*a*,*d*) are shown as mean values and standard errors. All experiments were repeated three times.

impairment of NEUROD1 binding to the *INS* promoter; or (ii) elimination of the function of the C-terminal transactivation domain of NEUROD1. We do not know whether these mutations in a heterozygous state might have disturbed the development of pancreatic islets and resulted in decreased β-cell mass in humans. In mice, targeted disruption of both alleles of *Neurod* results in abnormal islet morphology (including severely reduced β-cell mass and failure of islets to cluster), severe diabetes and perinatal death[1].

The explanation for the greater clinical severity in heterozygous carriers of the 206+C mutation compared with carriers of the R111L mutation is not clear. Although our functional studies showed that both mutations reduced insulin reporter expression in transient assays, it is uncertain whether this effect is the same *in vivo*. Moreover, the inability of the 206+C mutant to associate with p300 may have important implications for activation of islet genes during development. Target gene activation by p300 is mediated in part via an intrinsic histone acetyl transferase (HAT) activity that is proposed to function in chromatin remodelling[19,20]. We hypothesize that inability of the 206+C mutant to recruit such remodelling activity during development may block islet cell differentiation and reduce the number of functional β-cells in the islets of affected individuals.

Methods

Families. We recruited the families in this study for studies on the genetics of type 2 diabetes at the Joslin Diabetes Center[8,9]. Briefly, we selected and examined families because their pattern of occurrence of type 2 diabetes was consistent with an autosomal dominant mode of inheritance. An additional selection criterion was the availability of a large number of family members (with and without diabetes) willing to participate in the study. The screening criteria used to identify eligible families were: (i) an index case and at least 1 sibling having type 2 diabetes diagnosed between ages 10 and 60; (ii) the treatment for diabetes in the index case for the initial 2 years was diet or oral agents; and (iii) diabetes occurred in at least 3 generations. So far, we have recruited and examined 94 families of European descent. We examined, on average, 12 individuals per family (6 with and 6 without diabetes). Of the 94 families, 45 families had early onset type 2 diabetes that was considered to be MODY (median age at diagnosis in the family less than 40 years), and 49 families had diabetes diagnosed in middle age (between 40 and 59 years). The Committee on Human Sub-

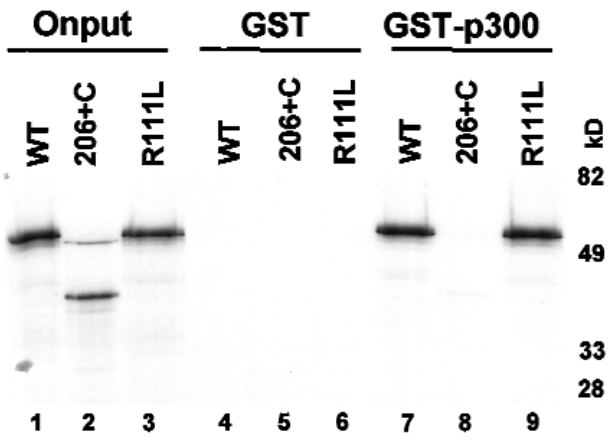


Fig. 6 Interaction of p300 with NEUROD1 mutants. ^{35}S-labelled, *in vitro* translated wild-type and mutant NEUROD1 proteins were examined for their capacity to interact with the previously defined C terminus (1,572–2,370) of cellular co-activator p300 expressed as a GST fusion protein. In contrast to the NEUROD1 206+C mutant (lane 8), NEUROD1-WT and NEUROD1-R111L mutant (lanes 7 and 9) interacted with the C terminus of p300 (aa 1,572–2,370). Lanes 1–3 show onput of the ^{35}S-labelled NEUROD1 proteins; note the faster migration of the truncated 206+C mutant protein. GST protein was used as a control (lanes 4–6). The migration of the molecular weight markers (kD) is shown. Experiments were repeated three times and showed similar results.

jects of the Joslin Diabetes Center approved the study protocol and informed consent procedures. After obtaining written consent to participate from individual family members, we measured their height and weight and drew fasting blood for blood glucose determination, DNA extraction and other biochemical measurements. From non-diabetic individuals and those diabetic individuals treated with oral agents or diet, we drew an additional blood sample 2 h after an oral challenge with glucose (75 g) for blood glucose determination and other biochemical measurements. We obtained fasting serum for C-peptide determination from insulin-treated individuals. All participants completed medical and family history questionnaires that were supplemented with information extracted from medical records. We calculated per cent ideal body weight (% of IBW) for males as 4.39×BMI and for females as 4.76×BMI (ref. 21). We used World Health Organization (WHO) criteria to diagnose diabetes and impaired glucose tolerance[22]. We selected 96 non-diabetic spouses of members of the families to examine the frequency of DNA sequence differences in *NEUROD1* in a control group.

Biochemical assays. Linco Research measured serum insulin and C peptide in family members and insulin in controls with impaired glucose tolerance by radioimmunoassay. The insulin assay showed little cross-reactivity (<2%) with human proinsulin. The Clinical Laboratory of the Joslin Diabetes Center performed the other measurements[9]. Radioligand assays for serum anti-GAD and anti-IA2 antibody levels used recombinant GAD and IA2 proteins and expressed results as CPM indexes[23,24]. The criterion for a positive assay for each antibody was an index greater than 0.1 (2 s.d. above the mean in normal controls).

Screening *NEUROD1* for sequence differences. We used three pairs of primers covering the entire coding sequence of the gene together with flanking sequences of *NEUROD1* (first pair, forward, 5′–CAAGCATTTGTACAGGTTTAG–3′, reverse, 5′–CTCCAGGCGAGCCTTAGTCATC–3′; second pair, forward, 5′–CCTCGAAGCCATGAACGCAG–3′, reverse, 5′–GCTGTCCATGGTACCGTAAG–3′; third pair, forward, 5′–CCTGCAACTCAATCCTCGGAC–3′, reverse, 5′–CTGTAAGCACAGTGGGTTCG–3′). We performed PCR reactions for each pair of specific primers using the following PCR conditions: denaturation at 95 °C for 5 min followed by 39 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, extension at 72 °C for 1 min and final extension for 10 min. The lengths of the PCR products were 407 bp, 583 bp and 561 bp, respectively. We purified the amplified DNA using QIAquick Gel Extraction Kit Protocol (Qiagen) and sequenced it directly from both ends using specific primers with AmpliTaq FS dye terminator cycle sequencing kit (PE Applied Biosystems). We performed electrophoresis on the ABI PRISM 377 DNA sequencer and used the ABI PRISM Sequencing Analysis software version 3.0 for the analysis. We confirmed the sequence of both mutations by cloning the PCR product of the entire coding sequence of the gene into a TA cloning vector (pCR 2.1, Invitrogen) and sequencing clones representing each allele.

Parametric linkage analysis. We performed parametric linkage analysis with LINKAGE software assuming an autosomal dominant mode of inheritance and a disease allele frequency of 0.001, consistent with the rarity of the mutations. For the susceptibility genotypes 'DD' and 'Dd', we assumed penetrances of 0.3, 0.5, 0.7 and 0.8 for the 4 age-related liability classes, 0–10, 11–25, 26–40 and over 40 years, respectively[25]. For the non-susceptible genotype 'dd', we used the risk of diabetes in the general population for the probabilities of diabetes in the same liability classes, 0.001, 0.005, 0.01 and 0.2, respectively.

Plasmids and vectors. We amplified *NEUROD1* wild-type and mutant cDNAs using the above clones as templates, and subcloned them in-frame into *Eco*R1 and *Hin*dIII sites of pBluescript. We verified the sequence and used the clones for *in vitro* expression of internally radiolabelled NEUROD1 proteins. For other constructs, we excised plasmids of the respective *NEUROD1* clones from pBluescript and cloned them into a PM1 (Clontech) vector for mammalian expression of Gal-NEUROD1 fusion proteins, pCDNA3-Flag for expression of Flag-tagged NEUROD1 proteins and PGEX-4T (Pharmacia) vector for bacterial expression of GST-NEUROD1 fusion proteins.

Gel shift assays. We incubated GST-NEUROD1 wild-type and mutant proteins and nuclear extracts from 293T cells transfected with CMV or CMV-E47 plasmids with 25,000 cpm of double-stranded RIPE3 oligonucleotide[2], end-labelled with [γ^{32}P]ATP using polynucleotide kinase. We carried out EMSA reactions for 20 min on ice in a final volume (20 µl) containing 20% glycerol, Hepes (20 mM, pH 7.6), EDTA (1 mM), KCl (50 mM), $MgCl_2$ (5 mM), BSA (1 µg), DTT (1 mM) and poly-dI-polydC (1 µg) as non-specific competitor. We resolved the complexes on a 4.5% non-denaturing polyacrylamide gels in 0.5×TBE (44.5 mM Tris, 44.5 mM borate and 1 mM EDTA).

Cell culture and transfections. We cultured HepG2 hepatoblastoma cells (ATCC) according to ATCC guidelines. Min6 cell cultures were maintained in DMEM, 15% FBS and β-mercaptoethanol (5 µM). We transfected the cells using Lipofectin (Life Technologies) according to the manufacturer's instructions, collected the cells after 35–40 h and assayed for CAT activity. We quantified CAT assays using a phosphoimager (Molecular Dynamics) and normalized to β-gal activity derived from a co-transfected CMV-β-Gal internal control plasmid. We assessed the comparability of expression of wild-type and mutant Flag-tagged and Gal-NEUROD1 proteins using pCDNA3 and PM-1 vectors, and western-blot analysis of nuclear extracts prepared from transfected Min6 and HepG2 cells using anti-Flag (Sigma) and anti-Gal antisera (Santa Cruz Biotechnology), respectively.

P300-NEUROD1 interactions. We prepared isopropyl-β-D-thiogalacto pyranoside (IPTG) induced cultures of *Escherichia coli* expressing the GST-P300 (aa 1,571–2,370) protein as described[22]. We internally labelled NEUROD1 wild-type and mutant proteins with ^{35}S-methionine *in vitro* using a TNT protein expression system (Promega). We incubated GST proteins (500 ng) with ^{35}S-labelled NEUROD1 proteins in a volume (400 µl) of NaCl (100 mM), EDTA (1 mM), Hepes (20 mM, pH 7.4), 0.5% NP-40 and DTT (1 mM). We complexed the GST and associated proteins to glutathione sepharose beads and resolved the complexes by SDS–PAGE.

GenBank accession numbers. *NEUROD1* sequence variants, U50822, AF045152.

Acknowledgements

We thank A. Leiter for GST-p300 constructs and helpful discussion. Funds from the National Institutes of Health grants DK-47475 (A.S.K.) and DK-36836 (Genetic Core of DERC at Joslin Diabetes Center) supported this project. A Juvenile Diabetes Foundation fellowship (JDF-39730) supported U.S.J.

Received 17 March; accepted 20 August 1999.

1. Naya, F.J. *et al*. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev.* **11**, 2323–2334 (1997).
2. Naya, F.J., Stellrecht, C.M. & Tsai, M.J. Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev.* **9**, 1009–1019 (1995).
3. Qiu, Y., Sharma, A. & Stein, R. p300 mediates transcriptional stimulation by the basic helix-loop-helix activators of the insulin gene. *Mol. Cell. Biol.* **18**, 2957–2964 (1998).
4. Sharma, A. *et al*. The NeuroD1/BETA2 sequences essential for insulin gene transcription colocalize with those necessary for neurogenesis and p300/CREB binding protein binding. *Mol. Cell. Biol.* **19**, 704–713 (1999).
5. Tamimi, R. *et al*. The NEUROD gene maps to human chromosome 2q32 and mouse chromosome 2. *Genomics* **34**, 418–421 (1996).
6. Yoon, Y.S., Noma, T., Yamashiro, Y., Ito, H. & Nakazawa, A. Molecular cloning and characterization of the gene encoding human NeuroD. *Neurosci. Lett.* **251**, 17–20 (1998).
7. Furuta H. *et al*. β-cell transcription factors and diabetes: mutations in the coding region of the BETA2/NeuroD1 (NEUROD1) and Nkx2.2 (NKX2B) genes are not associated with maturity-onset diabetes of the young in Japanese. *Diabetes* **47**, 1356–1358 (1998).
8. Ji, L. *et al*. New susceptibility locus for NIDDM is localized to human chromosome 20q. *Diabetes* **46**, 876–881 (1997).
9. Doria, A. *et al*. Phenotypic characteristics of early-onset, autosomal dominant type-2 diabetes unlinked to known MODY genes. *Diabetes Care* **22**, 253–261 (1999).
10. Dupont, S. *et al*. No evidence of linkage or diabetes-associated mutations in the transcription factors BETA2/NEUROD1 and PAX4 in type 2 diabetes in France. *Diabetologia* **42**, 480–484 (1999).
11. Yamagata, K. *et al*. Mutations in the hepatocyte nuclear factor-4α gene in the maturity-onset diabetes of the young (MODY1). *Nature* **384**, 458–460 (1996).
12. Yamagata, K. *et al*. Mutations in the hepatocyte nuclear factor-1α gene in maturity-onset diabetes of the young (MODY3). *Nature* **384**, 455–458 (1996).
13. Lehto, M. *et al*. Characterization of the MODY3 phenotype. Early-onset diabetes caused by an insulin secretion defect. *J. Clin. Invest.* **99**, 582–591 (1997).
14. Yokoyama, M. *et al*. Molecular cloning of a human neuroD from a neuroblastoma cell line specifically expressed in the fetal brain and adult cerebellum. *Brain Res. Mol. Brain Res.* **42**, 135–139 (1996).
15. Mutoh, H., Naya, F.J., Tsai, M.J. & Leiter, A.B. The basic helix-loop-helix protein BETA2 interacts with p300 to coordinate differentiation of secretin-expressing enteroendocrine cells. *Genes Dev.* **12**, 820–830 (1998).
16. Ma, P.C. *et al*. Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell* **77**, 451–459 (1994).
17. Ellenberger, T., Fass, D., Arnaud, M. & Harrison, S.C. Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. *Genes Dev.* **8**, 970–980 (1994).
18. Voronova, A. & Baltimore, D. Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. *Proc. Natl Acad. Sci. USA* **87**, 4722–4726 (1990).
19. Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H. & Nakatani, Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**, 953–959 (1996).
20. Yao, T.P. *et al*. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell* **93**, 361–372 (1998).
21. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. National Diabetes Data Group. *Diabetes* **28**, 1039–1057 (1979).
22. Harris, M.I., Hadden, W.C., Knowler, W.C. & Bennet, P.H. International criteria for the diagnosis of diabetes and impaired glucose tolerance. *Diabetes Care* **8**, 562–567 (1985).
23. Grubin, C.E. *et al*. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* **37**, 344–350 (1994).
24. Christie, M.R., Vohra, G., Champagne, D. & Delovitch, T.L. Distinct antibody specificities to a 64-kD islet cell antigen in type I diabetes as revealed by trypsin treatment. *J. Exp. Med.* **172**, 789–794 (1990).
25. Vaxillaire, M. *et al*. A gene for maturity onset diabetes of the young maps to chromosome 12q. *Nature Genet.* **9**, 418–423 (1995).