J Membrane Biol (2012) 245:263–273
DOI 10.1007/s00232-012-9447-1

Cx36 Is a Target of Beta2/NeuroD1, Which Associates with Prenatal Differentiation of Insulin-producing β Cells

Rachel Nlend Nlend · Aouatef Aït-Lounis · Florent Allagnat · Valentina Cigliola · Anne Charollais · Walter Reith · Jacques-Antoine Haefliger · Paolo Meda

Received: 28 March 2012 / Accepted: 1 June 2012 / Published online: 23 June 2012

Abstract The insulin-producing β cells of pancreatic islets are coupled by connexin36 (Cx36) channels. To investigate what controls the expression of this connexin, we have investigated its pattern during mouse pancreas development, and the influence of three transcription factors that are critical for β-cell development and differentiation. We show that (1) the Cx36 gene (*Gjd2*) is activated early in pancreas development and is markedly induced at the time of the surge of the transcription factors that determine β-cell differentiation; (2) the cognate protein is detected about a week later and is selectively expressed by β cells throughout the prenatal development of mouse pancreas; (3) a 2-kbp fragment of the *Gjd2* promoter, which contains three E boxes for the binding of the bHLH factor Beta2/NeuroD1, ensures the expression of Cx36 by β cells; and (4) Beta2/NeuroD1 binds to these E boxes and, in the presence of the E47 ubiquitous cofactor, transactivates the *Gjd2* promoter. The data identify Cx36 as a novel early marker of β cells and as a target of Beta2/NeuroD1, which is essential for β-cell development and differentiation.

Keywords Beta2/neurod1 · Connexin · Gap junctions · Gene regulation · Pancreas · Promoter · Transcription factor

R. N. Nlend · V. Cigliola · A. Charollais · P. Meda (✉)
Department of Cell Physiology and Metabolism, University of Geneva, CMU, 1 rue Michel Servet CH- 1211, Geneva 4, Switzerland
e-mail: paolo.meda@unige.ch

A. Aït-Lounis
Laboratory of Cellular and Molecular Biology, University of Algiers, USTHB, BP32 El-Alia, Bab Ezzouar, Algiers, Algeria

F. Allagnat · J.-A. Haefliger
Department of Internal Medicine, University of Lausanne, Bugnon 7A, 1005 Lausanne, Switzerland

W. Reith
Department of Pathology and Immunology, University of Geneva, CMU, 1 rue Michel Servet CH- 1211, Geneva 4, Switzerland

The insulin-producing β cells of pancreatic islets are coupled by Cx36 channels (Serre-Beinier et al. 2000; Theis et al. 2004). Although a previous report has documented how this connexin is repressed in most cell types (Martin et al. 2003), the mechanism that accounts for the consistent expression of Cx36 in β cells, neurons and neuron-derived cells (Bosco et al. 2011; Potolicchio et al. 2012) has not yet been investigated. In view of the changes in Cx36 distribution and levels which take place in different areas of the developing brain, and which correlate with critical differentiation steps of neuronal networks (Cina et al. 2007; Gulisano et al. 2000), we hypothesized that important insights about the obligatory expression of Cx36 in β cells could be gained by establishing the expression profile of the protein, as a function of the initiation, differentiation and growth of β cells (Herrera 2000), as well as the subsequent morphogenesis of pancreatic islets (Kim and MacDonald 2002). Therefore, we have studied the temporal and spatial expression of Cx36 during the pre- and postnatal development of the mouse pancreas. To this end, we used real-time PCR to evaluate the transcription of the Cx36 gene (*Gjd2*), and immunofluorescence to investigate the expression of the cognate protein during the development of mouse pancreas.

In view of the tight correlation between the expression of *Gjd2* and the two insulin genes (Carvalho et al. 2010; Serre-Beinier et al. 2009), we also hypothesized that the

expression of Cx36 may be controlled by the same set of transcription factors that regulate β-cell differentiation and function (Kim and MacDonald 2002; Habener et al. 2005; Murtaugh 2007; Jonsson et al. 1994; Naya et al. 1997; Zhang et al. 2005). Given the restricted distribution of Cx36 (Bosco et al. 2011; Potolicchio et al. 2012), we further posited that the expression of Cx36 was likely to be controlled by transcription factor or factors involved in the differentiation and function of both pancreatic β cells (Zhang et al. 2005) and neurons (Cho and Tsai 2004). Therefore, we investigated the effects on Cx36 expression of several transcription factors involved in pancreatic development (Herrera 2000; Kim and MacDonald 2002; Habener et al. 2005). To this end, we used a luciferase reporter system to test different regions of the *Gjd2* promoter, chromatin immunoprecipitation assays to evaluate the binding of Beta2/NeuroD1 to this promoter, and cell transfections, as well as site-directed mutagenesis, to assess the effects of Beta2/NeuroD1 on Cx36 expression. The data show that *Gjd2* is (1) expressed early in the development of the mouse pancreas, as reported in the chicken (Berthoud et al. 2004), and in a recent report (Pérez-Armendariz et al. 2012) published since the submission of this study; (2) encodes the cognate Cx36 protein at the time of the second wave of β-cell differentiation; (3) is a hitherto nonidentified target of Beta2/NeuroD1, which controls its expression by direct binding to the *Gjd2* promoter.

Materials and Methods

Animals

Adult C57Bl/6 mice (Charles Rivers, Lyon, France) were mated, and coupling confirmed by the presence of a vaginal plug on the next morning (E0.5). Embryos were then obtained from pregnant females at E11.5, E13.5, E15.5, and E17.5. Control mice were also sacrificed at stages P0 and P2, and after 1–2 months of life.

Histology

Cryostat sections (5-µm thickness) of pancreas were fixed for 10 min in 4 % paraformaldehyde (PFA) supplemented with 0.1 % Triton X-100. Sections were then incubated 30 min in 0.1 M phosphate-buffered saline (PBS) supplemented with 2 % BSA, and exposed for 2 h at room temperature to one of the following primary antibodies: rabbit polyclonal antibody anti-Cx36 (Zymed 36-4600), diluted 1/50; guinea pig polyclonal antibody anti-insulin (Ventrex Bioreagent 4043580), diluted 1/200; mouse monoclonal antibody anti-glucagon (Sigma G2654), diluted 1/2000. After rinsing, the sections were reacted with one of the following secondary antibodies, whichever appropriate: mouse anti-rabbit IgGs (Boehringer) conjugated to fluorescein isothiocyanate, diluted 1/400; goat anti guinea pig IgGs conjugated to rhodamine, diluted 1/200; goat anti-mouse IgGs conjugated to rhodamine (Boehringer), diluted 1/200. Immunolabeling was visualized under UV illumination.

RT-PCR

Total RNA was extracted from control mouse liver using Trizol (Invitrogen), and from pancreas (E11.5-P0) using the RNeasy micro kit (Qiagen), as recommended by the manufacturers. RNA of adult pancreas was extracted as previously reported (Serre-Beinier et al. 2000; Carvalho et al. 2010). Briefly, samples were exposed to 4 M guanidine isothiocyanate in the presence of 2 M β-mercaptoethanol and 2.5 mM Tris-HCl, layered on a 228 mM CsCl/0.1 M EDTA cushion (pH 7.4), and centrifuged at 35,000 rpm for 20 h at 20 °C. Pelleted RNA was resuspended in 10 mM Tris-HCl containing 5 mM EDTA and 0.1 % SDS. Samples were extracted three times with phenol–chloroform–isoamyl alcohol, and precipitated in ethanol. The dried pellets were resuspended in DEPC-H_2O and stored at −80 °C. Total RNA from Min6, INS1-E, and HeLa cells was extracted using Trizol (Invitrogen), as recommended by the manufacturer. The RNA quality was controlled using a laser Agilent 2100 bioanalyser. A total of 1 µg (standard PCR) or 750 ng (quantitative PCR) total RNA were reverse-transcribed using 200 U Reverse transcriptase Superscript II (Invitrogen), 10 ng/µl random hexamer primers (Promega) and 500 µM each dNTP mix (Invitrogen). The mixture was heated to 65 °C for 5 min and quickly chilled on ice. First strand buffer (5×), 10 mM dithiothreitol and 200 U Superscript II Reverse transcriptase were then added (all reagents purchased from Invitrogen). The samples were heated at 25 °C for 10 min, at 42 °C for 50 min and at 70 °C for a 15 min inactivation. 0.5 U RNase H (Roche) was then added for 20 min at 37 °C.

For real-time PCR, we used a 1:10 (cell lines)–1:16 (tissues) dilution of the cDNA in a mix containing 2× QuantiTect SYBR Green PCR Master Mix (1×; Qiagen) and 300 nM each primer. The amplification protocol consisted of a 10-min initial activation of the Taq polymerase at 95 °C, 40 cycles of denaturation at 95 °C for 15 s, and annealing at 65 °C for 1 min. The following primers were used: for Cx36, 5′-GAC CAT CTT GGA GAG GCT GC-3′ (F) and 5′-ACC ACC ACA GTC AAC AGG ATC C-3′ (R); for insulin2, 5′-GCA GGA AGC CTA TCT TCC AGG-3′ (F) and 5′-GGA CTC CCA GAG GAA GAG CAG-3′ (R); for Beta2/NeuroD1, 5′-ACG CAG AAG GCA AGG TGT C-3′ (F) and 5′-CCG CTC TCG CTG TAT

GAT TT-3′ (R); for Neurogenin3, 5′-AGA ACT AGG ATG GCG CCT CA-3′ (F) and 5′-GTG GCT AGG TGG GGT GGA A-3′ (R); for PDX1, 5′-AAG AGC CCA ACC GCG TC-3′ (F) and 5′-GTG TAA GCA CCT CCT GCC CA-3′ (R); for Cx32, 5′-AGT GCC AGG GAG GTG TGA AT-3′ (F) and 5′-ACA GCC ATA CTC GGC CAA T-3′ (R); for connexin43 (Cx43), 5′-ACT TTC ATT AAG TGA AAG AGA GGT GCC-3′ (F) and 5′-GCC GTC GAG TAG GCT TGG AG-3′ (R); for RSP9, 5′-GAC CAG GAG CTA AAG TTG ATT GGA-3′ (F) and 5′-TCT TGG CCA GGG TAA ACT TGA-3′ (R); for HPRT, 5′-GCT CGA GAT GTC ATG AAG GAG AT-3′ (F) and 5′-AAG AAC TTA TAG CCC CCC TTG A-3′ (R). A melting curve was run at the end of the 40 cycles to test for the presence of a unique PCR reaction product, using the following protocol: 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The PCR was performed using the 7900HT PCR system (Applied Biosystem). mRNA levels were normalized to those of the two ubiquitous genes *HPRT* and *RPS9*. The stable expression of these control genes in our samples was assessed using the Genorm software.

Bioinformatics

The sequences of mouse, rat and human Cx36 genes were compared using the multi-LAGAN function of the LAGAN Alignment Toolkit Web site (http://lagan.stanford.edu/lagan_web/ index.shtml). Conservation of response elements for Beta2/NeuroD1 was assessed using the Genomatix software (http://www.genomatix.de).

Cloning and Constructs

A −4,946/+485 fragment of *Gjd2* (the transcription start site being referred to as +1) was isolated from the mouse genomic library RP23-230H3 (Invitrogen), and cloned into a pGL3b luciferase reporter vector (Promega). A −2,010/−12 fragment was cloned in plasmid pCR4 (Invitrogen), and used as source of the promoter region. To this end, a *Sna*bI/*Xho*I fragment (−1,524/+474) of pCR4 was amplified by PCR using the Platinum Pfx DNA Polymerase (Invitrogen) and primers 5′-CTA TAC GTA GAA TCA CCG TCC CAT CTG TC-3′ (F) and 5′- CCG CTC GAG GGA GGC AGC AGA CAA AGA CT-3′ (R). The PCR product was first subcloned in TOPO vector pCR2.1 (Invitrogen), before being excised with SacI and XbaI, and inserted by ligation (T4 DNA ligase, Promega) into the reporter vector pGL3b (Promega), upstream of a cDNA coding for luciferase. Using the −1,524/+474 fragment as substrate, and primers 5′-AGA GTG CGG GAG TCC ATC-3′ (F) and 5′-CCG CTC GAG GGA GGC AGC AGA CAA AGA CT-3′ (R), we then generated a +192/+474 fragment, which was also subcloned in TOPO vector pCR2.1 (Invitrogen), excised with KpnI and XhoI, and inserted by ligation (T4 DNA ligase, Promega) into the reporter vector pGL3b (Promega), upstream of a cDNA coding for luciferase. Expression vectors for Beta2/NeuroD1 (pCMV-BETA2), its E47 coactivator (pCR3.1-E47) and the RIPE3 sequence (pINSCAT448) were generously provided by Dr. H. Suh-Kim (Kyonggi University, South Korea).

The mutated constructs 2Kb-E1m, 2Kb-E2m, and 2Kb-E3m were generated using as substrate the −2,010/−12 promoter construct, and the QuikChange Multi Site Directed Mutagenesis Kit (Stratagene), following the instructions of the manufacturer. The primers used for each construct were: for 2 Kb-E1 m, 5′-CGC GGG AGC GCT GGG TGC CCG CTC CAG TGA-3′ (F) and 5′ TCA CTG GAG CGG GCA CCC AGC GCT CCC GCG-3′ (R); for 2 Kb-E2 m, 5′-GCA GGA GCT CGG CTG CCT GCA CGC TGC C-3′ (F) and 5′-GGC AGC GTG CAG GCA GCC GAG CTC CTG C-3′ (R); for 2 Kb-E3 m, 5′-AGA CTG CGG GAG TCT GTC TGA CCC CCG G- 3′ (F) and 5′-CCG GGG GTC AGA CAG ACT CCC GCA GTC T-3′ (R).

Cell Culture

MIN6 and HeLa cells were grown at 37 °C, under 5 % CO_2, in Dulbecco's modified Eagle's medium (DMEM), containing 110 U/ml penicillin, 110 µg/ml streptomycin, and either 5 mM glucose plus 10 % heat-inactivated fetal calf serum (HeLa cells) or 25 mM glucose plus 15 % heat-inactivated FCS and 70 µM β-mercaptoethanol (MIN6 cells). INS-1E cells were grown at 37 °C, under 5 % CO_2, in RPMI-1640 medium containing L-glutamine, 10 mM Hepes, 5 % heat-inactivated fetal calf serum, 100 mM Na-Pyruvate, 5 mM β-mercapto-ethanol, 110 U/ml penicillin, and 110 µg/ml streptomycin

Transfection

Aliquots of 10^5 Min6, 10^5 INS1-E cells or 8×10^4 HeLa cells were plated per well in 12-well plates. The next day, cells were transfected using the Polyfect reagent (Qiagen), as per the manufacturer instructions. Each of the reporter pGL3b plasmids (1 µg) coding for a different Cx36 promoter fragment, was individually transfected with or without the plasmid pCMV-BETA2 (1 µg) coding for Beta2/NeuroD1, and plasmid pCR3.1-E47 (140 ng) coding for the E47 cofactor. In all experiments, 0.1 µg vector pRL-TK (Promega) coding for *Renilla* luciferase was cotransfected with these plasmids, to allow for a normalization of the firefly luciferase activity induced by the promoter reporter constructs. In each experiment, the total DNA amount was adjusted to the same level using an empty pCDNA.3 plasmid (Invitrogen).

Luciferase Assay

Two days after transfection, cells were lysed in PLB, and the activities of firefly (induced by the various pGL3b vectors) and Renilla luciferase (induced by the pRL-TK vector), were monitored using the Dual-luciferase reporter assay kit (Promega), as per the manufacturer instructions. To compare the basal activity of the various *Gjd2* fragments in the different cell lines, results are presented as mean + SEM luciferase activity, normalized to that of the Renilla reporter. To compare the Beta2/NeuroD1 regulation of the *Gjd2* fragments in each cell line, we assessed luciferase activity of cells cotransfected with plasmid pCMV-BETA2 (coding for Beta2/NeuroD1), and plasmid pCR3.1-E47 (coding for the E47 cofactor) or only with a control empty vector. Data are shown as means + SEM, normalized to the luciferase activity of the respective promoter fragment, which was transfected with the empty vector.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed as previously described (Masternak et al. 2003). Briefly, crosslinked chromatin was prepared by exposing 4×10^7 Min 6 cells to 1 % formaldehyde for 8 min at room temperature. Crosslinking was stopped by the addition of 0.2 M glycine. The cells were then lysed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing protease inhibitors and 0.5 % NP-40. Nuclei were pelleted and lysed in TE containing 0.5 M NaCl, 1 % Triton X-100 and 0.5 % sodium deoxycholate. Crosslinked chromatin was resuspended in TE buffer supplemented with 100 mM NaCl, sheared by sonication, and cleared by two successive centrifugations, first at 4,000 rpm for 10 min and then at 13,000 rpm for 15 min. One aliquot of 10 µg sheared chromatin was saved for the input, while another 10-µg aliquot was used for immunoprecipitation at 4 °C overnight, using 2 µg rabbit polyclonal antibody against NeuroD1 (Abgent AP2021b). The immune complexes were captured at room temperature using protein A-Sepharose beads (GE Healthcare Biosciences).The immunoprecipitated chromatin fragments were eluted for 10 min at 65 °C with 100 mM Tris-HCl containing 1 % SDS (pH 8), digested for 2 h at 42 °C with 150 µg/ml Proteinase K, and incubated overnight at 65 °C to reverse the crosslinks. After extraction with phenol-chloroform and chloroform, DNA was precipitated with isopropanol in the presence of glycogen, washed in 75 % ethanol and resuspended in 50 µl TE buffer. The immunoprecipitated DNA and the input chromatin DNA were analyzed by qPCR using a SYBR-Green kit (Eurogentec) and the following primers: for the Cx36 promoter, 5′-CCTCAGACCGCAA GATCG-3′ (F) and 5′-TGG GGG AAG CAA TCT ATG TG-3′ (R); for the insulin promoter, 5′-TGT TGA CGT CCA ATG AGC GCT TTC-3′ (F) and 5′-TA G GTC AGC AGA TGG CCA GAG G-3′ (R); for TG737, 5′- GCC AAG GCT ACA CAA GAC-3′ (F) and 5′-CAC CAG TGC TTC CGA TTC-3′ (R). A standard curve was generated with the input chromatin, and binding of Beta2/NeuroD1 to the Cx36 promoter was evaluated relative to that of the same transcription factor to the insulin promoter, which was used as positive control.

Statistical Analysis

Student's *t* tests were used for statistical analysis throughout the study. p values <0.05 were considered as statistically significant.

Results

Gjd2 Transcription Starts during Early Pancreas Development and Changes at Selected Time Points

To determine the temporal pattern of Cx36 expression, we amplified RNA extracted from the pancreas of wild type mice, age E11.5 onward. Real-time quantitative PCR showed that the levels of Cx36 mRNA increased ($p < 0.005$) 12-fold between E11.5 and E15.5, and by a further 1.6-fold ($p < 0.01$) from E15.5 to P0 (Fig. 1a). Thereafter, these levels apparently decreased (Fig. 1a) as a result of the dilution of the endocrine transcripts in the total pancreas extracts, as a result of the explosive, postnatal expansion of the exocrine pancreas. This was confirmed by the postnatal 3.5 increase in Cx32, a connexin isoform expressed by the acinar cells of pancreas (Fig. 1a). The mRNA levels of this connexin, as well as those of the Cx43 isoform, which is expressed in the nonendocrine and non-acinar compartments of the pancreas, featured an expression pattern quite different from that of Cx36, during both pre- and postnatal development of pancreas (Fig. 1a).

The first peak of Cx36 expression occurred at the prenatal time (E15.5) of the so called "secondary transition" (Pictet et al. 1972), during which β cells massively differentiate, as revealed by increased transcription of the insulin gene (*Ins;* Fig. 1b). The second peak of Cx36 expression (P0) also occurred at the time of increased transcription of *Ins,* which reached adult levels within the first 2 days of neonatal life (Fig. 1b). Quantitative PCR further showed that the increase in Cx36 expression between E13.5 and E15.5 coincided with that of Beta2/NeuroD1, PDX1 (Fig. 1b) and Ngn3 (data not shown), three transcription factors which are obligatory for proper pancreas development and β-cell function (Itkin-Ansari et al. 2005; Naya et al. 1995; Offield et al. 1996).

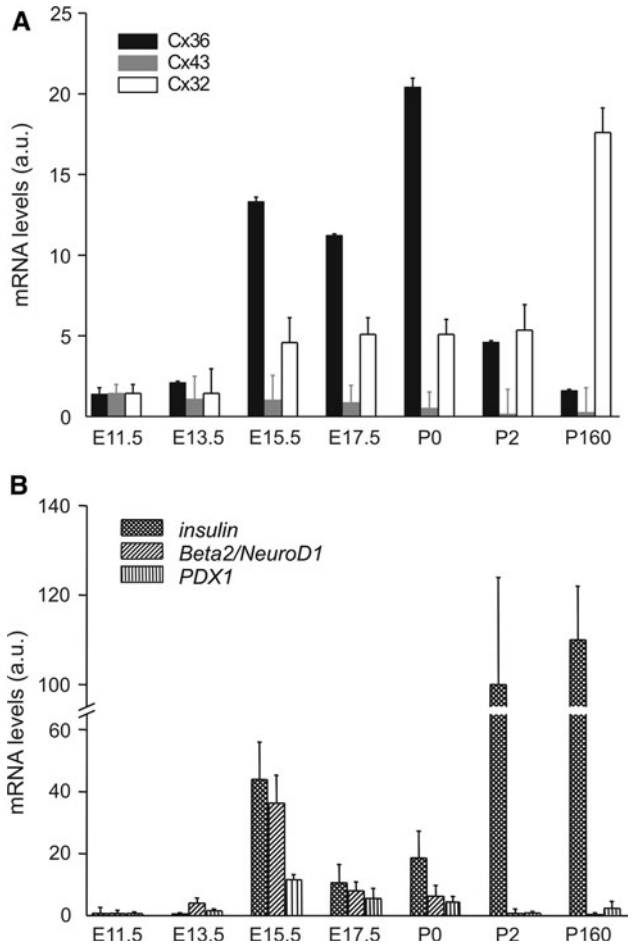


Fig. 1 *Gjd2* transcription is detected in early pancreas development, and changes at selected time points. **a** Real-time PCR showed that the levels of the Cx36 mRNA (*solid columns*) increased sharply between E13.5 and E15.5, and at birth (P0), in contrast to those of both Cx43 (*grey bars*) and Cx32 transcript (*open bars*). **b** Prenatal and neonatal increase in the levels of the insulin mRNA (*cross-hatched bars*) was coincident with that of Cx36 mRNA (*solid bars* in **a**). The increased transcription of Cx36 between E13.5 and E15.5 (*solid bars* in **a**) was also coincident with that of Beta2/NeuroD1 (*diagonally hatched bars*) and PDX1 (*vertically hatched bars*). Bars represent the mean + SEM fold change in mRNA level, relative to the level evaluated at E11.5; $n = 4$ for E13.5–E17.5; $n = 5$ for E11.5 and P times

Thereafter, the levels of these factors decreased up to the adult age (Fig. 1b) as a result of the increasing growth of the exocrine tissue. These experiments show that the expression of Cx36 is initiated during early pancreas development, and follows a temporal pattern which is different from that of other connexins and which is initiated at the time the expression of the insulin gene and of major β-cell transcription factors surges.

To identify the cell types expressing Cx36, we immunostained the pancreas of control C57Bl6 mice. No Cx36 was detected in the pancreas of E11.5, E13.5, and E15.5 mice, when few cells contained detectable levels of either insulin (Fig. 2) or glucagon (data not shown). Later on, Cx36 spots were found on insulin-containing (Fig. 2), but were never observed in glucagon-containing cells (data not shown). These experiments show that Cx36 is expressed during the early development of the mouse pancreas, and later becomes a specific attribute of β cells.

A Fragment of the *Gjd2* Promoter Ensures the Specific Expression of Cx36 in Insulin-producing Cells

To unravel the mechanism controlling the expression of Cx36, we generated luciferase reporter plasmids coding for fragments of the *Gjd2* promoter, and transiently transfected lines of the insulin-producing Min6 and INS1-E cells, as well as the nonendocrine line of HeLa cells. When compared to the void vector, the 282-bp promoter fragment induced a similar increase ($p < 0.001$) in luciferase activity in the three cell types (Fig. 3a). The 2- and 5-kbp fragments also significantly increased ($p < 0.001$) luciferase activity over that seen in cells transfected with the void vector (Fig. 3a). However, this effect was significantly larger ($p < 0.01$) in the two insulin-producing lines than in HeLa cells (Fig. 3a). The results indicate that the 2-kbp fragment of the *Gjd2* promoter is sufficient to ensure a specific expression of Cx36 in insulin-producing cells, and that the region comprised between −2,010 and −294 bp contains elements that regulate its expression level.

In silico analysis identified in this region three E boxes with a CANNTG sequence characteristic of cis-elements binding bHLH factors (Sommer et al. 1996; Robinson et al. 2000), which were referred to as E1, E2 and E3, at position −280, −332, and −445, respectively (Fig. 3b). Comparison of the sequences of these E boxes of mouse, rat and human *Gjd2,* showed a highly conservation of the canonic CANNTG sequence, especially in E1 and E3 (Fig. 3c).

A Fragment of the *Gjd2* Promoter Binds Beta2/NeuroD1, Which Transactivates Cx36 Expression

Beta2/NeuroD1 is a bHLH transcription factor central to the development and differentiation of both β cells (Herrera 2000; Itkin-Ansari et al. 2005; Naya et al. 1995a; Offield et al. 1996; Sommer et al. 1996; Robinson et al. 2000; Naya et al. 1994, 1995) and neurons (Masternak et al. 2003; Lee 1995, 1997; Liu et al. 2000; Poulin et al. 1997), which binds to E boxes to regulate the expression of the insulin gene (*Ins;* Itkin-Ansari et al. 2005; Naya et al. 1995). To test whether Beta2/NeuroD1 also binds to *Gjd2* promoter, we run chromatin immunoprecipitation assays on Min6 cells. We first validated the protocol by assessing the immunoprecipitated chromatin using primers for the promoter of *Ins* (Fig. 4a) which, thereafter, was used as normalization standard. Relative to this standard, the binding of Beta2/NeuroD1 to the region of the *Gjd2*

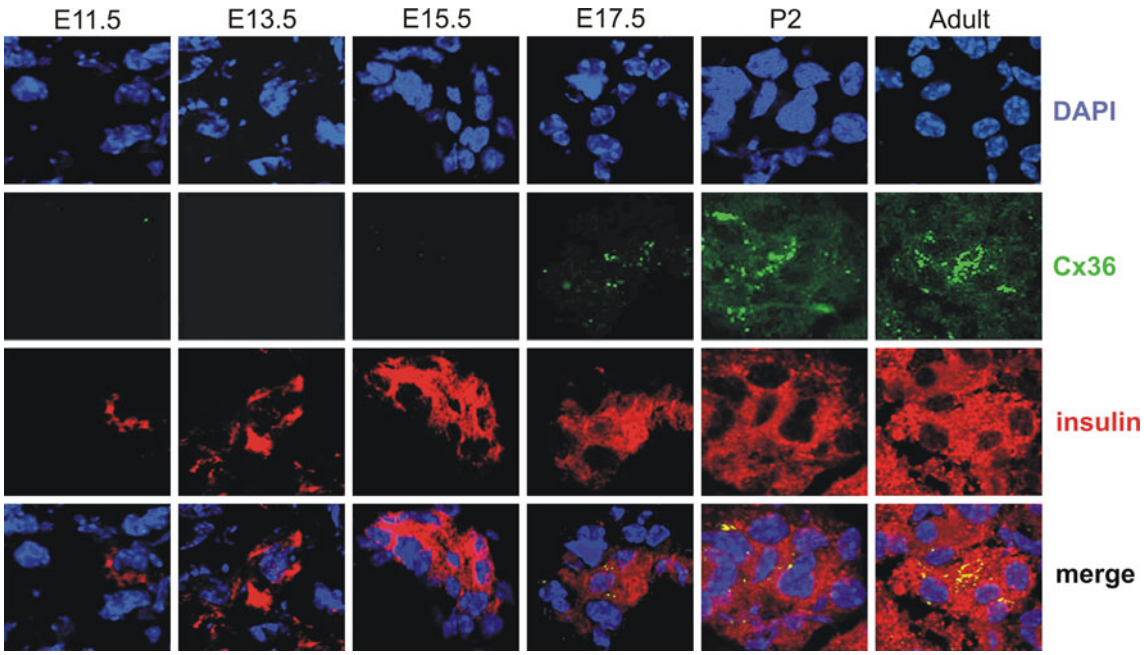


Fig. 2 The Cx36 protein is detected in β cells during late pancreas development. The same area of a control mouse pancreas is shown after staining of cell nuclei (DAPI, *blue*), Cx36 (*green*), and insulin (*red*). Cx36 was immunodetected from E17.5 onward and showed a punctate distribution only in insulin-containing β cells (*red*). *Bars* 15 μm

promoter that contained E boxes represented 43 ± 15 % (Fig. 4a). This binding was significantly higher ($p < 0.05$) than the nonspecific binding of the transcription factor to either a downstream region of the *Gjd2* promoter or to a region of the promoter of the unrelated *TG737,* which served as negative controls (Fig. 4a). The results show that Beta2/NeuroD1 specifically binds to the region of the *Gjd2* promoter containing the three E boxes.

To investigate whether this binding modulated the expression of Cx36, we cotransfected Beta2/NeuroD1, together with the ubiquitous cofactor E47 which is required for its activity (Qiu et al. 2002; Mirasierra and Vallejo 2006; Kim et al. 2002; Docherty et al. 2005), and one of the 5.4-kbp, 2-kbp, or 282-bp *Gjd2* promoter constructs (Fig. 4a) in Min6, INS1-E and HeLa cell lines. Although the former two insulin-producing cell types natively express Beta2/NeuroD1, the latter does not (Fig. 4b) and thus provided for a negative control. The parallel transfection of the RIPE3-luc construct, which contained three multimerized copies of the E box of the rat *Ins* promoter upstream of a luciferase reporter sequence (Kim et al. 2002; Henderson and Stein 1994), provided for a positive control. Relative to the basal luciferase activity observed in cells that had not been transfected with Beta2/NeuroD1 and E47, cells transfected for these factors plus the void pGL3b vector did not show a change in luciferase activity (Fig. 4c). In contrast, transfection of the RIPE3 construct resulted in a significant increase ($p < 0.01$) in luciferase activity in the three cell types (Fig. 4c). Under these conditions, which validated the experimental protocol, the 282-bp fragment of *Gjd2,* which contained the E3 box, did not significantly modify the luciferase activity (Fig. 4c). In contrast, this activity was significantly increased ($p < 0.005$) over basal values after transfection of either the 2-kbp or the 5.4-kbp fragments of *Gjd2,* which contained the E1, E2 and E3 boxes (Fig. 4c). These results show that Beta2/NeuroD1 transactivates the promoter of *Gjd2* via cis-elements of the 5.4-kbp and the 2-kbp fragments, possibly E1 and E2.

To test the role of these sequences, each of the three E boxes was mutated by scrambling the consensus core sequence CANNTG in the 2-kbp fragment of the *Gjd2* promoter, which was chosen because it provided for the largest effect of Beta2/NeuroD1 (Fig. 4c). Thus, four mutated versions of the 2-kbp promoter were constructed, three carrying a single mutated E box and one carrying a combined mutation of the three E boxes (Fig. 4d). The mutated constructs were then tested in INS1-E and HeLa cells after cotransfection with Beta2/NeuroD1 and E47. Compared to the luciferase activity of the wild-type 2-kbp construct, the activity observed after mutation of E1 was significantly reduced ($p < 0.05$), whereas that observed after a mutation of either E2 or E3 had a minimal effect (Fig. 4d). The effect of the E1 mutation was larger ($p < 0.005$) in the presence of a concurrent mutation of E2 and E3 (Fig. 4d). The results indicate that Beta2/NeuroD1 transactivates *Gjd2* via the native E boxes of its promoter.

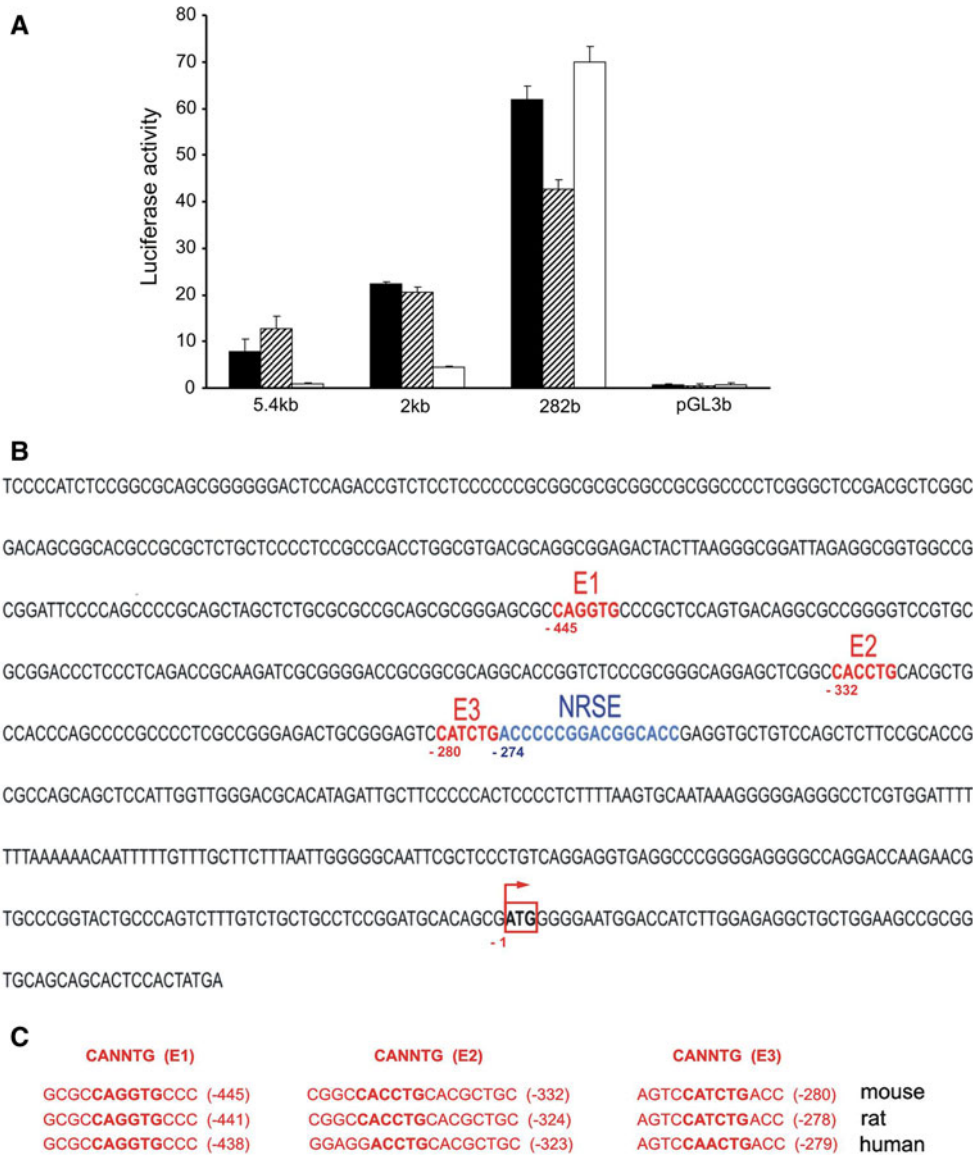
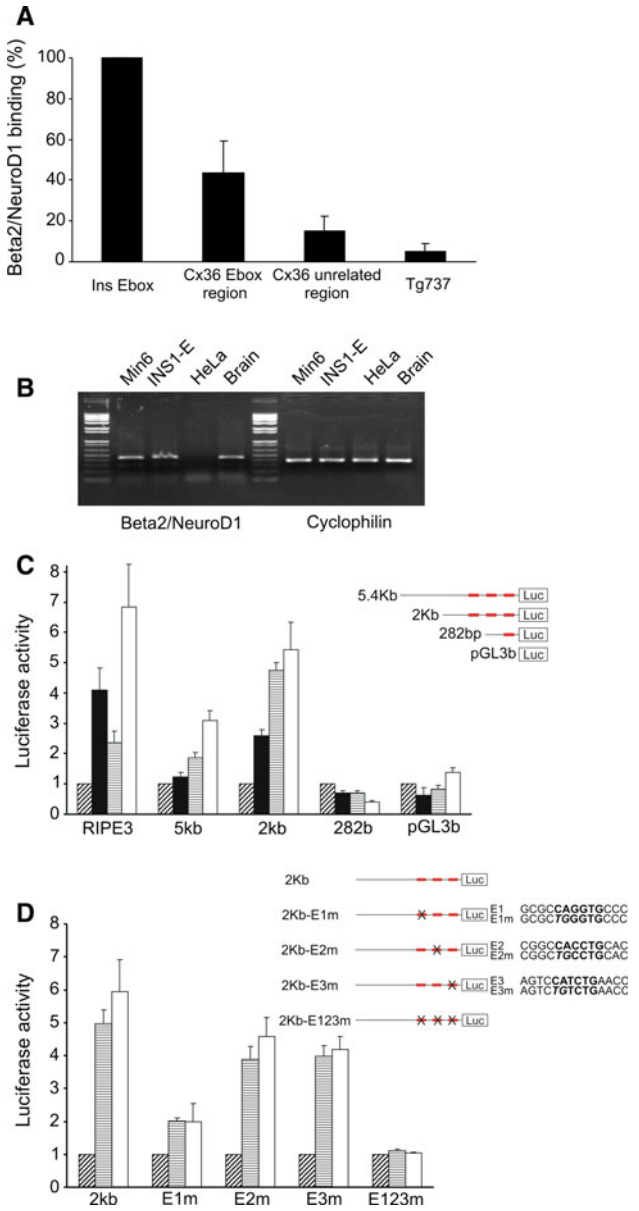


Fig. 3 A promoter fragment ensures the specific expression of *Gjd2* in insulin-producing cells. **a** Three fragments of the *Gjd2* promoter were transiently transfected, using a luciferase reporter plasmid. The 282-bp fragment increased luciferase activity over that provided by an empty pGL3b vector in MIN6 (*solid bars*), INS1E (*hatched bars*), and HeLa cells (*open bars*). The 2-kbp and 5-kbp constructs induced a smaller increase, which was restricted to the insulin-producing MIN6 and INS1E cells. Values are mean + SEM of 3 experiments and are shown after normalization to the luciferase activity observed in cells transfected with pRL-TK. **b** The partial sequence of the *Gjd2* promoter shows the position (relative to that of the translation start site, which is referred to as +1) of the 3 E boxes (*red*). The previously identified NRSE (Martin et al. 2003) is shown in *blue*. **c** Alignment of the mouse, rat, and human Cx36 promoters shows the conservation of the consensus CANNTG sequence of the 3 E boxes

Discussion

We have investigated the pattern of Cx36 expression during pancreas development and the differentiation of pancreatic β cells. Our data confirm that *Gjd2* is transcribed during the early prenatal development of pancreas (Berthoud et al. 2004; Pérez-Armendariz et al. 2012), before the first wave of β cells can be detected (Theis et al. 2004; Herrera 2000; Berthoud et al. 2004; Pérez-Armendariz et al. 2012). Using antibodies, we have further shown that Cx36 becomes detectable at later stages of the prenatal pancreas development, but only in cells containing immunodetectable insulin. Cx36 largely increases in these cells during the neonatal period, when β cells acquire their major differentiation characteristic, i.e. the ability to recognize circulating glucose as a stimulus for insulin secretion (Gulisano et al. 2000; Herrera 2000; Carvalho et al. 2010, 2012). This evolution is consistent with the previous findings that Cx36 is required for the proper functioning of differentiated islets (Ravier et al. 2005; Speier et al. 2007; Klee et al. 2011), and provides the first clue that this requirement actually precedes the functional maturation of these microorgans. Strikingly, immunolabeling showed no association between Cx36 and the glucagon-containing α-cells, extending previous biochemical, immunological and functional observations (Serre-Beinier et al. 2000; Theis et al. 2004; Bosco et al. 2011; Kim and MacDonald 2002), which indicated that, throughout rodent life, Cx36 expression and signalling are solely restricted to the β-cell lineage.

The initial surge of *Gjd2* occurred at the time (E13.5–E15.5), the first wave of β cells is induced (Herrera 2000; Kim and MacDonald 2002; Habener et al. 2005), as evidenced by the concomitant induction of the insulin gene.



◀ **Fig. 4** Beta2/NeuroD1 binds to E boxes and transactivates the *Gjd2* promoter. **a** DNA of Min6 cells was immunoprecipitated using antibodies to Beta2/NeuroD1, then amplified by qPCR. Beta2/NeuroD1 bound to the region of the *Gjd2* promoter containing the E boxes more than to a Gjd2 region devoid of E boxes and to the unrelated *Tg737* promoter. Data are mean + SEM from 3 independent experiments and are expressed relative to the binding of Beta2/NeuroD1 to the *Ins* promoter, which served as positive control. **b** RT-PCR showed the native expression of Beta2/NeuroD1 in MIN6 and INS1E cells, but not in HeLa cells. Brain served as positive control. **c** After transient cotransfection with Beta2/NeuroD1 and E47, the 282-bp fragment of the *Gjd2* promoter did not increase luciferase activity of Min6 (*solid bars*), INS1-E (*horizontally hatched bars*), and HeLa cells (*open bars*), over the basal level observed in nontransfected cells (*obliquely hatched bars*), whereas the 2-kbp and the 5.4-kbp fragments did. The RIPE3 and the void pGL3B plasmids served as positive and negative controls, respectively. Values are mean + SEM of 3 experiments and are expressed relative to the luciferase activity observed in cells transfected with pRL-TK. **d** After transient cotransfection with Beta2/NeuroD1 and E47, the 2-kbp fragment of *Gjd2* increased the luciferase activity of INS1-E cells (*horizontally hatched bars*) and HeLa cells (*open bars*) over the basal level observed in nontransfected cells (*obliquely hatched bars*). This effect was decreased after mutation of E1, blocked after the combined mutation of the 3 E boxes, but unaffected after mutation of either E2 or E3. Values are mean + SEM luciferase activity of 3 experiments, relative to the basal luciferase activity evaluated in the absence of exogenous Beta2/NeuroD1 (*obliquely hatched bars*)

This coincidence was not observed for either Cx32 or Cx43, showing that the pattern of Cx36 expression is specifically controlled at key moments of β-cell development. In turn, this timing suggests that Cx36 may be implicated in this development and/or be regulated by the very same transcription factors that determine pancreas differentiation. Quantitative PCR indeed documented that the prenatal *Gjd2* expression increases at the same time of the expression surge of the major transcription factors which contribute to the specification and differentiation of pancreatic β cells (Herrera 2000; Kim and MacDonald 2002; Habener et al. 2005). Previous studies have shown that the RE-1 silencing transcription factor (REST) prevents the expression of *Gjd2* in non-insulin-producing and nonneuronal cells (Martin et al. 2003; Hohl and Thiel 2005). However, no transcription factor was so far identified as a transactivator of the expression of this gene. Our data show that a 2-kbp region of mouse *Gjd2* contains cis-regulatory elements that are sufficient to determine the expression of Cx36 in insulin-producing cells. We further document that this region allows for the binding of the transcription factor Beta2/NeuroD1 to a specific E box (E1) of the *Gjd2* promoter. In the presence of its coactivator E47, Beta2/NeuroD1 transfection resulted in the transactivation of this promoter, which was potentiated by the two close-by E2 and E3 boxes. The extent of the transactivation of the 2-kbp and 5.4-kbp fragments induced by Beta2/NeuroD1 was lower in the insulin-producing cells than in the unrelated HeLa cells, suggesting that endogenous Beta2/NeuroD1 resulted in a higher basal transcriptional activity of *Gjd2* in the former cell type. Consistent with this hypothesis, mutation of E1 resulted in loss of the *Gjd2* transactivation dependent on the expression of transfected Beta2/NeuroD1, but not of the basal activity of the Cx36 promoter in INS-1E cells (data not shown). This finding implies that multiple factors converge to regulate the expression of the Cx36 gene in insulin-producing cells, as is the case for the insulin gene *Ins,* which is selectively regulated by the combinatorial effect of Beta2/NeuroD1, PDX1, and MafA transcription factors (Aramata et al. 2005). The *Gjd2* promoter contains putative binding sites for PDX1 (data not shown). However, these sites are not highly conserved, in spite of the consistent β cells expression of Cx36 in all species so far investigated

(Serre-Beinier et al. 2000; Theis et al. 2004; Pérez-Armendariz et al. 2012; Le Gurun et al. 2003), raising concerns that they may be dispensable for Cx36 expression.

An implication of these results is that some of the effects attributable to Beta2/NeuroD1 signaling, which has been shown to be essential for the terminal differentiation and survival of the β cells (Habener et al. 2005; Naya et al. 1995a), may be mediated by Cx36. The validation of this tentative conclusion awaits further studies on the molecular mechanism linking the timely related changes in the expression of these two proteins. Strikingly, mice lacking Beta2/NeuroD1 die around birth, with severe diabetes, ketoacidosis (Naya et al. 1997), and neuronal alterations (Miyata et al. 1999). Future studies should therefore evaluate whether altered Cx36 expression may be implicated in these defects. The previous reports that mice lacking this connexin show altered β-cell function (Carvalho et al. 2010; Ravier et al. 2005; Speier et al. 2007; Klee et al. 2011; Wellershaus et al. 2008) is consistent with this view. The observation that these animals do not feature major alterations in the development of islets and β-cell differentiation (Ravier et al. 2005; Klee et al. 2011; Wellershaus et al. 2008; Degen et al. 2004) only indicates that Cx36 is but one of the many actors that participate in the complex regulation of islet development and β-cell function (MacDonald 2007; MacDonald and Rorsman 2007). Thus, loss of Cx36 signaling may be compensated by other mechanisms, as documented in neurons (Cummings et al. 2008). The key importance of Beta2/NeuroD1 in pancreas development and function is further stressed by the observations that mutations inactivating the Beta2/NeuroD1 gene cause one of the rare monogenic forms of diabetes, referred to as MODY6 (Malecki et al. 1999), and that several variants of this gene confer susceptibility to the much more frequent type I and type II forms of diabetes (Iwata et al. 1999; Malecki et al. 2003; Yamada et al. 2001). The mechanism linking the genetic mutations to the various forms of the disease remains unknown. However, together with the reports that mice lacking Cx36 develop structurally normal islets which cannot sustain normal insulin secretion (Ravier et al. 2005; Speier et al. 2007; Klee et al. 2011; Wellershaus et al. 2008; Head et al. 2012; Meda 2012), the clinical data implicate that the predominant effect of the regulation of Cx36 expression by Beta2/NeuroD1 is likely on the function, rather than on the mass of the insulin-producing β cells (Martin et al. 2003; Bosco et al. 2011; Head et al. 2012; Meda 2012).

Acknowledgments Our team is supported by Grants from the Swiss National Science Foundation (310000-141162, IZ73Z0_127935, CR32I3_129987), the Juvenile Diabetes Research Foundation (40-2011-11, 5-2012-281), and the European Union (BETAIMAGE 222980; IMIDIA C2008-T7, BETATRAIN 289932). We are pleased to dedicate this study to Ross G. Johnson on occasion of the starting of his new life. Ross has been a pioneer in our field, a passionate and influential scholar, and a much appreciated host while Paolo was striving in snowy Minneapolis. We wish him all the best for a new exciting adventure.

Reference

Aramata S, Han SI, Yasuda K, Kataoka K (2005) Synergistic activation of the insulin gene promoter by the beta-cell enriched transcription factors MafA, Beta2, and Pdx1. Biochim Biophys Acta 1730:41–46

Berthoud VM, Singh R, Minogue PJ, Ragsdale CW, Beyer EC (2004) Highly restricted pattern of connexin36 expression in chick somite development. Anat Embryol 209:11–18

Bosco D, Haefliger JA, Meda P (2011) Connexins: key mediators of endocrine function. Physiol Rev 91:1393–1445

Carvalho CP, Barbosa HC, Britan A, Santos-Silva JC, Boschero AC, Meda P, Collares-Buzato CB (2010) Beta cell coupling and connexin expression change during the functional maturation of rat pancreatic islets. Diabetologia 53:1428–1437

Carvalho CP, Oliveira RB, Britan A, Silva-Santos JC, Boschero AC, Meda P, Collares-Buzato CB (2012) Impaired beta-to-beta cell coupling mediated by Cx36 gap junctions in pre-diabetic mice. Am J Physiol Endocrinol Metab 302 (in press)

Cho JH, Tsai MJ (2004) The role of BETA2/NeuroD1 in the development of the nervous system. Mol Neurobiol 30:35–47

Cina C, Bechberger JF, Ozog MA, Naus CC (2007) Expression of connexins in embryonic mouse neocortical development. J Comp Neurol 504:298–313

Cummings DM, Yamazaki I, Cepeda C, Paul DL, Levine MS (2008) Neuronal coupling via connexin36 contributes to spontaneous synaptic currents of striatal medium-sized spiny neurons. J Neurosci Res 86:2147–2158

Degen J, Meier C, Van Der Giessen RS, Sohl G, Petrasch-Parwez E, Urschel S, Dermietzel R, Schilling K, De Zeeuw CI, Willecke K (2004) Expression pattern of lacZ reporter gene representing connexin36 in transgenic mice. J Comp Neurol 473:511–525

Docherty HM, Hay CW, Ferguson LA, Barrow J, Durward E, Docherty K (2005) Relative contribution of PDX-1, MafA and E47/beta2 to the regulation of the human insulin promoter. Biochem J 389:813–820

Gulisano M, Parenti R, Spinella F, Cicirata F (2000) Cx36 is dynamically expressed during early development of mouse brain and nervous system. Neuroreport 11:3823–3828

Habener JF, Kemp DM, Thomas MK (2005) Minireview: transcriptional regulation in pancreatic development. Endocrinology 146:1025–1034

Head WS, Orseth ML, Nunemaker CS, Satin LS, Piston DW, Benninger RK (2012) Connexin-36 gap junctions regulate in vivo first- and second-phase insulin secretion dynamics and glucose tolerance in the conscious mouse. Diabetes 61 (in press)

Henderson E, Stein R (1994) c-jun inhibits transcriptional activation by the insulin enhancer, and the insulin control element is the target of control. Mol Cell Biol 14:655–662

Herrera PL (2000) Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 127:2317–2322

Hohl M, Thiel G (2005) Cell type-specific regulation of RE-1 silencing transcription factor (REST) target genes. Eur J Neurosci 22:2216–2230

Itkin-Ansari P, Marcora E, Geron I, Tyrberg B, Demeterco C, Hao E, Padilla C, Ratineau C, Leiter A, Lee JE, Levine F (2005) NeuroD1 in the endocrine pancreas: localization and dual function as an activator and repressor. Dev Dyn 233:946–953

Iwata I, Nagafuchi S, Nakashima H, Kondo S, Koga T, Yokogawa Y, Akashi T, Shibuya T, Umeno Y, Okeda T, Shibata S, Kono S, Yasunami M, Ohkubo H, Niho Y (1999) Association of polymorphism in the NeuroD/BETA2 gene with type 1 diabetes in the Japanese. Diabetes 48:416–419

Jonsson J, Carlsson L, Edlund T, Edlund H (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371:606–609

Kim SK, MacDonald RJ (2002) Signaling and transcriptional control of pancreatic organogenesis. Curr Opin Genet Dev 12:540–547

Kim JW, Seghers V, Cho JH, Kang Y, Kim S, Ryu Y, Baek K, Aguilar-Bryan L, Lee YD, Bryan J, Suh-Kim H (2002) Transactivation of the mouse sulfonylurea receptor I gene by BETA2/NeuroD. Mol Endocrinol 16:1097–1107

Klee P, Allagnat F, Pontes H, Cederroth M, Charollais A, Caille D, Britan A, Haefliger JA, Meda P (2011) Connexins protect mouse pancreatic β cells against apoptosis. J Clin Invest 121: 4870–4879

Le Gurun S, Martin D, Formenton A, Maechler P, Caille D, Waeber G, Meda P, Haefliger JA (2003) Connexin-36 contributes to control function of insulin-producing cells. J Biol Chem 278: 37690–37697

Lee JE (1997) NeuroD and neurogenesis. Dev Neurosci 19:27–32

Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H (1995) Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 268:836–884

Liu M, Pereira FA, Price SD, Chu MJ, Shope C, Himes D, Eatock RA, Brownell WE, Lysakowski A, Tsai MJ (2000) Essential role of Beta2/NeuroD1 in development of the vestibular and auditory systems. Genes Dev 14:2839–2854

MacDonald MJ (2007) Synergistic potent insulin release by combinations of weak secretagogues in pancreatic islets and INS-1 cells. J Biol Chem 282:6043–6052

MacDonald PE, Rorsman P (2007) The ins and outs of secretion from pancreatic beta-cells: control of single-vesicle exo- and endocytosis. Physiology (Bethesda) 22:113–121

Malecki MT, Jhala US, Antonellis A, Fields L, Doria A, Orban T, Saad M, Warram JH, Montminy M, Krolewski AS (1999) Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. Nat Genet 23:323–328

Malecki MT, Cyganek K, Klupa T, Sieradzki J (2003) The Ala45Thr polymorphism of Beta2/NeuroD1 gene and susceptibility to type 2 diabetes mellitus in a Polish population. Acta Diabetol 40: 109–111

Martin D, Tawadros T, Meylan L, Abderrahmani A, Condorelli DF, Waeber G, Haefliger JA (2003) Critical role of the transcriptional repressor neuron-restrictive silencer factor in the specific control of connexin36 in insulin-producing cell lines. J Biol Chem 278:53082–53089

Masternak K, Peyraud N, Krawczyk M, Barras E, Reith W (2003) Chromatin remodeling and extragenic transcription at the MHC class II locus control region. Nat Immunol 4:132–137

Meda P (2012) The in vivo β-to-β-cell chat room: connexin connections matter. Diabetes 61 (in press)

Mirasierra M, Vallejo M (2006) The homeoprotein Alx3 expressed in pancreatic beta-cells regulates insulin gene transcription by interacting with the basic helix-loop-helix protein E47. Mol Endocrinol 20:2876–2889

Miyata T, Maeda T, Lee JE (1999) NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes Dev 13:1647–1652

Murtaugh LC (2007) Pancreas and beta-cell development: from the actual to the possible. Development 134:427–438

Naya FJ, Stellrecht CM, Tsai MJ (1994) Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev 9:1009–1919

Naya FJ, Stellrecht CM, Tsai MJ (1995) Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev 9:1009–1019

Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ (1997) Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev 11:2323–2334

Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV (1996) PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development 122:983–995

Pérez-Armendariz EM, Cruz-Miguel L, Coronel-Cruz C, Esparza-Aguilar M, Pinzon-Estrada E, Rancaño-Camacho E, Zacarias-Climaco G, Olivares PF, Espinosa AM, Becker I, Sáez JC, Berumen J, Pérez-Palacios G (2012) Connexin 36 is expressed in beta and connexins 26 and 32 in acinar cells at the end of the secondary transition of mouse pancreatic development and increase during fetal and perinatal life. Anat Rec 295:980–990

Pictet RL, Clark WR, Williams RH, Rutter WJ (1972) An ultrastructural analysis of the developing embryonic pancreas. Dev Biol 29:436–467

Potolicchio I, Cigliola V, Velazquez-Garcia S, Klee P, Valjevac A, Kapic D, Cosovic E, Lepara O, Hadzovic-Dzuvo A, Mornjacovic Z, Meda P (2012) Connexin-dependent signaling in neurohormonal systems. Biochim Biophys Acta 1818:1919–1936

Poulin G, Turgeon B, Drouin J (1997) NeuroD1/beta2 contributes to cell-specific transcription of the proopiomelanocortin gene. Mol Cell Biol 17:6673–6682

Qiu Y, Guo M, Huang S, Stein R (2002) Insulin gene transcription is mediated by interactions between the p300 coactivator and PDX-1, BETA2, and E47. Mol Cell Biol 22:412–420

Ravier MA, Guldenagel M, Charollais A, Gjinovci A, Caille D, Sohl G, Wollheim CB, Willecke K, Henquin JC, Meda P (2005) Loss of connexin36 channels alters beta-cell coupling, islet synchronization of glucose-induced Ca^{2+} and insulin oscillations, and basal insulin release. Diabetes 54:1798–1807

Robinson KA, Koepke JI, Kharodawala M, Lopes JM (2000) A network of yeast basic helix-loop-helix interactions. Nucleic Acids Res 28:4460–4466

Serre-Beinier V, Le Gurun S, Belluardo N, Trovato-Salinaro A, Charollais A, Haefliger JA, Condorelli DF, Meda P (2000) Cx36 preferentially connects beta-cells within pancreatic islets. Diabetes 49:727–734

Serre-Beinier V, Bosco D, Zulianello L, Charollais A, Caille D, Charpantier E, Gauthier BR, Diaferia GR, Giepmans BN, Lupi R, Marchetti P, Deng S, Buhler L, Berney T, Cirulli V, Meda P (2009) Cx36 makes channels coupling human pancreatic beta-cells, and correlates with insulin expression. Hum Mol Genet 18:428–439

Sommer L, Ma Q, Anderson DJ (1996) Neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. Mol Cell Neurosci 8:221–241

Speier S, Gjinovci A, Charollais A, Meda P, Rupnik M (2007) Cx36-mediated coupling reduces beta-cell heterogeneity, confines the stimulating glucose concentration range, and affects insulin release kinetics. Diabetes 56:1078–1086

Theis M, Mas C, Doring B, Degen J, Brink C, Caille D, Charollais A, Kruger O, Plum A, Nepote V, Herrera P, Meda P, Willecke K (2004) Replacement by a lacZ reporter gene assigns mouse connexin36, 45 and 43 to distinct cell types in pancreatic islets. Exp Cell Res 294:18–29

Wellershaus K, Degen J, Deuchars J, Theis M, Charollais A, Caille D, Gauthier B, Janssen-Bienhold U, Sonntag S, Herrera P, Meda P, Willecke K (2008) A new conditional mouse mutant reveals

specific expression and functions of connexin36 in neurons and pancreatic beta-cells. Exp Cell Res 314:997–1012

Yamada S, Motohashi Y, Yanagawa T, Maruyama T, Kasuga A, Hirose H, Matsubara K, Shimada A, Saruta T (2001) NeuroD/beta2 gene G→A polymorphism may affect onset pattern of type 1 diabetes in Japanese. Diabetes Care 24:1438–1441

Zhang C, Moriguchi T, Kajihara M, Esaki R, Harada A, Shimohata H, Oishi H, Hamada M, Morito N, Hasegawa K, Kudo T, Engel JD, Yamamoto M, Takahashi S (2005) MafA is a key regulator of glucose-stimulated insulin secretion. Mol Cell Biol 25: 4969–4976