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Islet-1 Regulates *Arx* Transcription during Pancreatic Islet α-Cell Development*

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Aristaless related homeodomain protein (Arx) specifies the formation of the pancreatic islet α-cell during development. This cell type produces glucagon, a major counteracting hormone to insulin in regulating glucose homeostasis in adults. However, little is known about the factors that regulate *Arx* transcription in the pancreas. In this study, we showed that the number of Arx$^+$ cells was significantly reduced in the pancreata of embryos deficient for the Islet-1 (Isl-1) transcription factor, which was also supported by the reduction in *Arx* mRNA levels. Chromatin immunoprecipitation analysis localized Isl-1 activator binding sites within two highly conserved noncoding regulatory regions (Re) in the *Arx* locus, termed Re1 (+5.6 to +6.1 kb) and Re2 (+23.6 to +24 kb). Using cell line-based transfection assays, we demonstrated that a Re1- and Re2-driven reporter was selectively activated in islet α-cells, a process mediated by Isl-1 in overexpression, knockdown, and site-directed mutation experiments. Moreover, *Arx* mRNA levels were upregulated in islet α-cells upon Isl-1 overexpression *in vivo*. Isl-1 represents the first known activator of *Arx* transcription in α-cells, here established to be acting through the conserved Re1 and Re2 control domains.

Pancreatic islets play an important role in regulating carbohydrate metabolism through the production and secretion of hormones. The five cell types found in the islet are α-, β-, δ-, ϵ-, and pancreatic polypeptide cells that, respectively, produce the hormones glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide (1). The hormonal products of the predominant α- and β-cells act in peripheral tissues in a counter-regulatory manner to control blood glucose homeostasis, with insulin promoting cellular glucose uptake and storage and glucagon promoting its release. Notably, although insulin resistance and β-cell dysfunction are the major causes of diabetes, the sustained, unregulated secretion of glucagon from α-cells also contributes to hyperglycemia and the associated complications (2–6). In fact, suppression of glucagon activity or levels has been shown to be a promising treatment for diabetics (3, 6–9). Unfortunately, and in contrast to islet β-cells, our understanding of the factors involved in controlling α-cell differentiation and function is quite limited (1, 10).

Glucagon$^+$ cells first appear at embryonic day (E) 9.5[3] in mice, followed by insulin$^+$ cells a day later (1). At around E13.5, a major expansion of a distinct population of hormone-producing cells begins to occur, with only these cells becoming mature islet α- and β-cells (11). A variety of distinct transcription factors are required during development for their production (1), including those that are necessary early in specifying the α- (*e.g.* Arx) and β- (*e.g.* Pax4 and Nkx6.1) lineages and others acting later in cell maintenance and maturation (*e.g.* MafB, Foxa2, Isl-1, and Pdx1) (12–20).

Arx plays an essential role in islet α-cell formation. Expression of this transcription factor is first detected in the pancreatic anlage at E9.5, then in differentiating pancreatic endocrine precursors, and subsequently in islet α-cells (15). *Arx*-null or pancreas-specific *Arx*-deficient mice display a complete loss of α-cells and a proportional increase in the number of β- and δ-cells (16, 21). Such changes are opposite to those found in *Pax4*-null mice, a transcription factor enriched in β-cell progenitors and islet β-cells (16, 20). Importantly, Pax4 and Arx function in an antagonistic manner and are both critical in the specification of these islet cell types during pancreatic development (15). For example, Arx misexpression in the pancreatic epithelium specifically converted β-cells into α- and pancreatic polypeptide cells, whereas *Pax4* produced more β-cells at the expense of the α-cell population (22, 23). Significantly, as islet α-cells were also lost in humans with an *ARX*-null mutation (24), the collective evidence strongly suggests that this transcription factor serves as a master regulator of mammalian islet α-cell formation.

Thus far, the only established regulator of *Arx* transcription in the pancreas is Pax4, which inhibits expression through binding to a *cis*-acting element located ~14.2 kb downstream of the *Arx* translation stop site (15). Recently, we have shown that removal of the Lin 1/Islet 1/Mec3-homeodomain Islet-1 (Isl-1) transcription factor in the pancreatic epithelium reduced both α- and β-cell numbers as well

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[3] The abbreviations used are: E9.5, embryonic day 9.5; Isl-1, Islet 1; Re, region(s); HD, homeodomain.

as key transcriptional regulators associated with their identity; specifically, MafA and Arx in β- and α-cells, respectively (17). These observations suggested that Isl-1 might be a good candidate for their regulation. The goal of this study was to investigate whether *Arx* is directly regulated by Isl-1 in α-cells. We identified two highly conserved regulatory regions (Re) within the *Arx* locus, termed Re1 and Re2, that are bound by Isl-1 and promote expression selectively in α-cells. Furthermore, we provide evidence that Isl-1 activates *Arx* transcription using a combination of *in vitro* and *in vivo* based loss-of-function and gain-of-function experiments. These results establish Isl-1 as a key activator of *Arx* transcription during α-cell formation and potentially also as a critical regulator for its regulation in adults.

EXPERIMENTAL PROCEDURES

Isl-1 Chromatin Immunoprecipitation Assays—Approximately 800 to 1000 islets were isolated from five 8-week-old CD1 male mice using the standard collagenase procedure as described previously (25). The islets were washed in PBS, cross-linked in 1.1% formaldehyde, and sonicated in buffer containing 50 mM Tris-HCl (pH8.1), 10 mM EDTA, 0.05% SDS, and complete protease inhibitor mixture (Roche). αTC6 (26) and βTC3 tissue-cultured cells were cross-linked under similar conditions, lysed in hypotonic buffer (50 mM Tris-HCl, 0.5% Nonidet P-40, 85 mM KCl with protease inhibitor mixture (Roche)), and sonicated. α-Isl-1 antibody (Developmental Studies Hybridoma Bank at the University of Iowa, 39.4D5) was added to the sonicated chromatin, and Isl-1 bound chromatin was isolated by incubation with protein A-agarose beads and eluted in buffer containing 1% SDS and 0.1 M Na_2CO_3. The immunoprecipitated DNA was purified using a PCR purification kit (Qiagen) and analyzed by either ChIP-Seq or real-time PCR analysis. ChIP PCR primer sequences are as follows: *ArxRe1*, 5′CCATTTGAAGGCAAAATGCT and 5′GTATGGGCTGCAAACACCTT; *ArxRe2*, 5′TGAAGTGGCTGAATGAGAGC and 5′AGTTGGAGCGCGTTTTGTAG; and *PEPCK*, 5′CAACAGGCAGGGTCAAAGTTTAG and 5′AGGCCTCAGGCCCCTCTAT.

Whole-genome ChIP-Seq Analysis—The purified immunoprecipitated DNA was prepared for next-generation high-throughput sequencing using the ChIP-Seq DNA sample prep kit (Illumina, San Diego, CA) following the manufacturer's instructions. The modified product was assayed for quantity and quality on an Agilent 2100 bioanalyzer (Agilent Tech). Sequencing and data processing were performed at the Diabetes Endocrinology Research Center. Functional Genomics Core at University of Pennsylvania. Isl-1-bound regions designated by peaks were identified using the Global Identifier of Target Regions algorithm (27).

Real-time PCR Analysis—RNA was isolated from islets or tissue-cultured cells using the RNAeasy kit (Qiagen), and cDNA was generated using Oligo(dT), Superscript II reverse transcriptase, plus the accompanying reagents (Invitrogen). Real-time PCR reactions were set up using the Brilliant SYBR Green PCR Master Mix (Stratagene). All reactions were performed in triplicate with reference dye for normalization. PCR primer sequences are as follows: Arx, 5′TCAAGCATAGCCGCGCTGAG and 5′ACACCTCCTTCCCCGTGCTG and Isl-1, 5′CGGAGAGACATGATGGTGGTT and 5′GGGCTGATCTATGTCGCTTTGC.

Luciferase Vector Construction and Reporter Assays—Arx Re1, and Re2 were cloned into the p*GL4.27* luciferase vector (Promega). Site-directed mutants were generated with the QuikChange mutagenesis kit (Stratagene) to create Isl-1 binding site mutations (underlined). The corresponding wild-type nucleotide sequences for these sites are labeled in red in Fig. 5, *A* and *B*: Re1–1, 5′CCGCCCTGCACAAGGAGCTGGCGCCGGCTATTTGATTTCC; Re1–3, 5′GGGAGTGAGTGCAATGCTGCCGAAGGTGTTTGCAGCCC; Re2–2, 5′CAATAACAAGCATGGCCGTAAGTGAGCATGATGAAGTTAATGG; Re2–3, 5′GTGAGCATGATGAAGTGCCGGGAGATAATTCATTCC; and Re2–6, 5′GCATAATTGCAGAGAATGAAGCCGTGATTTGACAATTGTACCAG.

αTC1–6 cells were cultured in 12-well plates (1×10^5 cells/well) and transfected with the *Arx*-driven expression vectors as well as the *Renilla* luciferase control expression plasmid (*pRL-SV40*) using Lipofectamine 2000 reagent (Invitrogen). Reporter assays were performed using the dual-luciferase reporter assay system (Promega) following the manufacturer's instructions. *Arx*-luciferase activity was normalized to that of *Renilla* luciferase.

Lentiviral Transduction—Lentiviral transduction particles (MISSION® shRNA, Sigma) targeting Isl-1 were applied to cultured αTC1–6 cells according to the manufacturer's instructions (multiplicity of infection = 5). Infected cells were selected for puromycin (5 μg/ml) resistance for 1 week before analysis. The Isl-1 shRNA sequence is as follows: 5′CCGGCGGCAATCAAATTCACGACCACTCGAGTGGTCGTGAATTTGATTGCCGTTTTTG.

Western Blotting—Whole cell lysate from control and Isl-1 shRNA transduced αTC1–6 cells as well as isolated islets (equivalent of ~200 islets) were loaded and resolved on 10% Bis-Tris NuPAGE® Novex® mini gels (Invitrogen). The antibodies used for the Western blot analysis were Isl-1 (1:1000, 39.4D5, Developmental Studies Hybridoma Bank at the University of Iowa), tubulin (1:5000, Sigma), and Myc (1:1000, Cell Signaling Technology).

Immunofluorescence—Tissues were fixed in 4% paraformaldehyde overnight at 4 °C and then embedded in paraffin. Slides (8–10-μm sections) were subjected to microwave antigen retrieval in 10 mM citric acid buffer (pH 6.0) and treated with protein blocking reagent (Immunotech). Slides were incubated with primary antibodies overnight at 4 °C and secondary antibodies for 2 h at room temperature. Primary antibodies used were insulin (1:1000, Linco), glucagon (1:3000, Linco), Myc (1:100, Cell Signaling Technology), and Arx (1:2000, gift from Dr. Kitamura at Kyushu University, Japan).

EMSA—DNA binding reactions (20 μl total volume) included 10 μg of αTC1–6 nuclear extract (28) or 2 μl of *in vitro* translated Isl-1-Myc protein (17) plus 400 fmol of ^{32}P end-labeled DNA oligo probe. Reactions were incubated at 4 °C in a buffer containing 10 mM HEPES (pH 7.9), 75 mM KCL, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 3% (v/v) Ficoll, 1 mg/ml BSA, 500 ng poly(dI-dC), and 500 ng of poly(dA-dT). Competition experiments were performed using a 100-fold molar excess of the unlabeled oligonucleotide. α-Isl-1 (Devel-

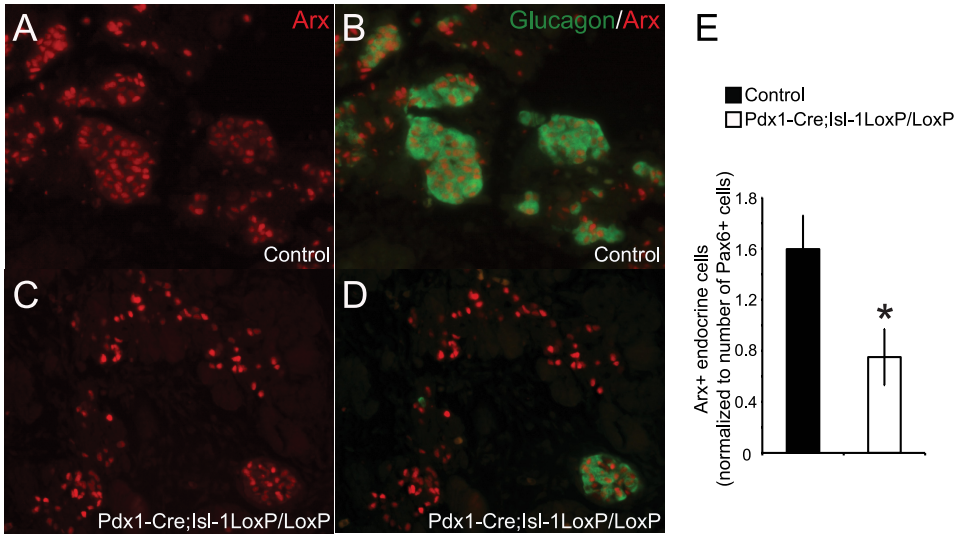

FIGURE 1. **The number of Arx$^+$ cells is reduced in the *Pdx1-Cre;Isl- 1$^{LoxP/LoxP}$* embryos.** *A–D*, immunostaining with glucagon and Arx antibodies of E15.5 pancreatic sections from control and *Pdx1-Cre;Isl-1$^{LoxP/LoxP}$* embryos. *E*, quantitative analysis of Arx$^+$ cell populations as normalized to Pax6$^+$ cells in the neighboring section. $n = 6$ for control and $n = 5$ for mutants. Data represents the mean ± S.E. *, p value <0.05.

opmental Studies Hybridoma Bank at the University of Iowa, 40.2D6) or α-Myc antibody (SC-40, Santa Cruz Biotechnology) was incubated with the nuclear extract for 10 min on ice prior to probe addition in the supershift assays. Reaction products were separated on 6% non-denaturing acrylamide gels in 0.5× TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) buffer, and complexes were visualized by autoradiography. The double-stranded oligonucleotide sequences are as follows, with the putative homeodomain (HD) binding elements in bold lettering: Re1-1wild-type, 5′GAGCTGGC**TAAT**GCTATTTG; Re1-2wild-type, 5′CATTGATC**TAAT**AGGGAGTG; Re1-3wild-type, 5′GCAATGCT**ATTA**AAGGTGTT; Re1-4wild-type, 5′-GGCTCATT**TAAT**ACACAACA; Re1-5wild-type, 5′GCTTCTTGC**ATTA**GAGGGC; Re1-6wild-type: 5′GAGGGC**TAAT**GATGAGGCTG; Re2–1wild-type, 5′AGAAGCAC**ATTA**TTTCTTTT; Re2–2wild-type, 5′AAGCATG**TAATTA**AGTGAGC; Re2–2mutant, 5′AAGCATGGCCGGCAGTGAGC (mutation is underlined); Re2–3wild-type, 5′GATGAAGT**TAAT**GGAGATAC; Re2–3mutant, 5′GATGAAGTGCCGGGAGATAC (mutation is underlined); Re2–4wild-type, 5′AGCTGT**TAAT**TTAATCATCAT; Re2–5wild-type, 5′TGCGAGCA**TAAT**TGCAGAGA; Re2–6wild-type, 5′AGAATGAA**TAAT**TGATTTGA; and Re2–6mutant, 5′AGAATGAAGCCGTGATTTGA (mutation is underlined).

Transgene Construction and Generation of Pdx1^{PB}-Isl-1-Myc Mice—The *Hnf6* cDNA (from *Pdx1^{PB}-Hnf6,* gift from M. Gannon, 29) was replaced by rat *Isl-1-Myc* (gift from Sam Pfaff). DNA was SalI-digested, and the entire *Pdx1^{PB}-Isl-1-Myc* transgene was injected into the pronuclei of C57BL6J embryos at the Children's Hospital of Philadelphia Transgenic Core Facility. Founder mice were identified by PCR analysis with the following genotyping primers: 5′CCCCTCTGCTAACCATGTTC and 5′TACCGCAACCAACACATAGG.

RESULTS

Arx$^+$Cell Numbers Were Reduced in Isl-1-deficient Pancreata—Mice with pancreas-specific removal of Isl-1 were generated in our lab previously to investigate the requirement of Isl-1 during pancreas development (17). *Arx* mRNA levels were found previously to be decreased in the embryonic *Pdx1-Cre;Isl-1$^{LoxP/LoxP}$* pancreata, conditions wherein removal of Isl-1 in pancreatic epithelium at E13.5 also resulted in reduced numbers of insulin$^+$ and glucagon$^+$ cells (17). In contrast, the steady-state levels of all other analyzed islet-enriched transcriptional regulators were unaffected, with the exception of MafA in insulin$^+$ cells (17). Immunostaining for glucagon and Arx was performed with E15.5 control and *Pdx1-Cre;Isl-1$^{LoxP/LoxP}$* pancreata to examine whether Arx$^+$ cells were affected in the mutant (Fig. 1, *A-D*). Pax6 staining was performed on neighboring sections and used as an internal control for quantitative analysis because the mRNA expression level and number of Pax6$^+$ cells were unaltered in the E15.5 *Pdx1-Cre;Isl-1$^{LoxP/LoxP}$* pancreas (17).

From this analysis, we found that the total number of Arx$^+$ cells was reduced by ~50% in the *Pdx1-Cre;Isl-1$^{LoxP/LoxP}$* pancreata (Fig. 1*E*), with the few remaining glucagon$^+$ cells coexpressing Arx (Fig. 1*D*). Unlike the control pancreas, most of the Arx$^+$ cells in the mutant pancreas did not produce glucagon, likely representing the significance of Isl-1 in activating glucagon production (17). Here we have focused on understanding how Isl-1 affects *Arx* mRNA expression in islet α-cells.

Transgenic Isl-1 Expression Increased Arx Expression in Mice—To further examine Isl-1 regulation in islet α-cell gene expression *in vivo*, a transgenic line was derived that expressed rat *Isl-1-Myc* from the Pst1 to Bst1 region of the mouse *pdx-1* promoter, termed *Pdx1^{PB}-Isl-1-Myc* (Fig. 2*A*). The transgenic activity of this *pdx-1* control region is first observed at around E13.5 and is essential only in islet α, β, and δ cells throughout adulthood (29, 30). Isl-1 mRNA and protein levels were increased ~2-fold in 8-week old *Pdx1^{PB}-Isl-1-Myc* mice relative to age-matched controls (Fig. 2, *B* and *C*). Double immunostaining in islets for Myc and glucagon or insulin revealed that Isl-1-Myc was produced in 4% of glucagon$^+$ and 32% insulin$^+$ cells (Fig. 2, *D–G*). This *pdx-1* fragment has been shown

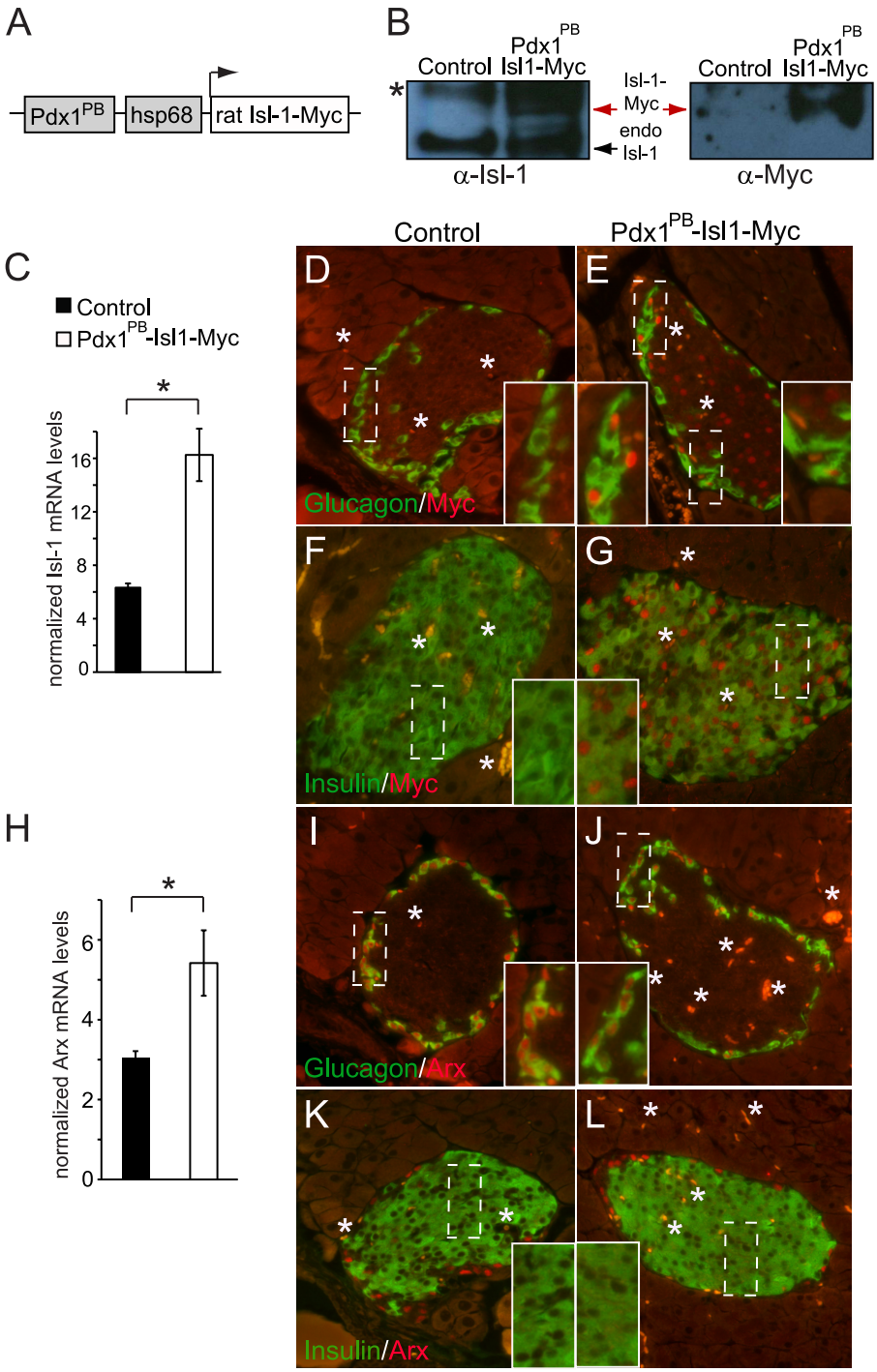

FIGURE 2. **Arx expression is elevated *Pdx1^{PB}-Isl-1-Myc* mice.** *A*, diagram representing the *Pdx1^{PB}-Isl-1-Myc* transgenic construct, which contains the *pdx1^{PB}* fragment of the *pdx1* gene and the *hsp68* minimal promoter. *B*, Western blot by α-Isl-1 and α-Myc analysis of total cell lysate from islets of 8-week-old control and *Pdx1^{PB}-Isl-1-Myc* mice. The *asterisk* denotes a nonspecific band. *C*, *Isl-1* mRNA levels in *Pdx1^{PB}-Isl-1-Myc* and littermate control mice. Results are presented as mean ± S.E. *, $p < 0.05$. *D–G*, immunohistochemical analysis for glucagon or insulin and Myc expression in 8-week-old *Pdx1^{PB}-Isl-1-Myc* and control mice. *H*, *Arx* mRNA levels in *Pdx1^{PB}-Isl-1-Myc* and control mice. Results are presented as mean ± S.E. *, $p < 0.05$. *I–L*, costaining of Arx with glucagon or insulin in 8-week-old *Pdx1^{PB}-Isl-1-Myc* and control mice. *Insets* show the magnified views of the outlined areas. The *asterisk* denotes autofluorescence from red blood cells.

previously to drive stronger transgene expression in islet β-cells than α-cells (29).

Pdx1^{PB}-Isl-1-Myc mice appeared indistinguishable from their control littermates at 8 weeks of age. In addition, we did not detect any gross abnormalities in the overall appearance of the pancreas. Immunohistochemical analysis of *Pdx1^{PB}-Isl-1-Myc* pancreata showed no changes in islet morphology, size, or the ratio between glucagon- and insulin-expressing cells (Fig. 2, *D–L*). Furthermore, mRNA levels of glucagon and insulin were also comparable between *Pdx1^{PB}-Isl-1-Myc* and control littermates (data not shown).

Interestingly, Isl-1-Myc overexpression elevated endogenous *Arx* mRNA levels by roughly 2-fold (Fig. 2*H*). Notably, Arx protein staining was only found in *Pdx1^{PB}-Isl-1-Myc* α-cells

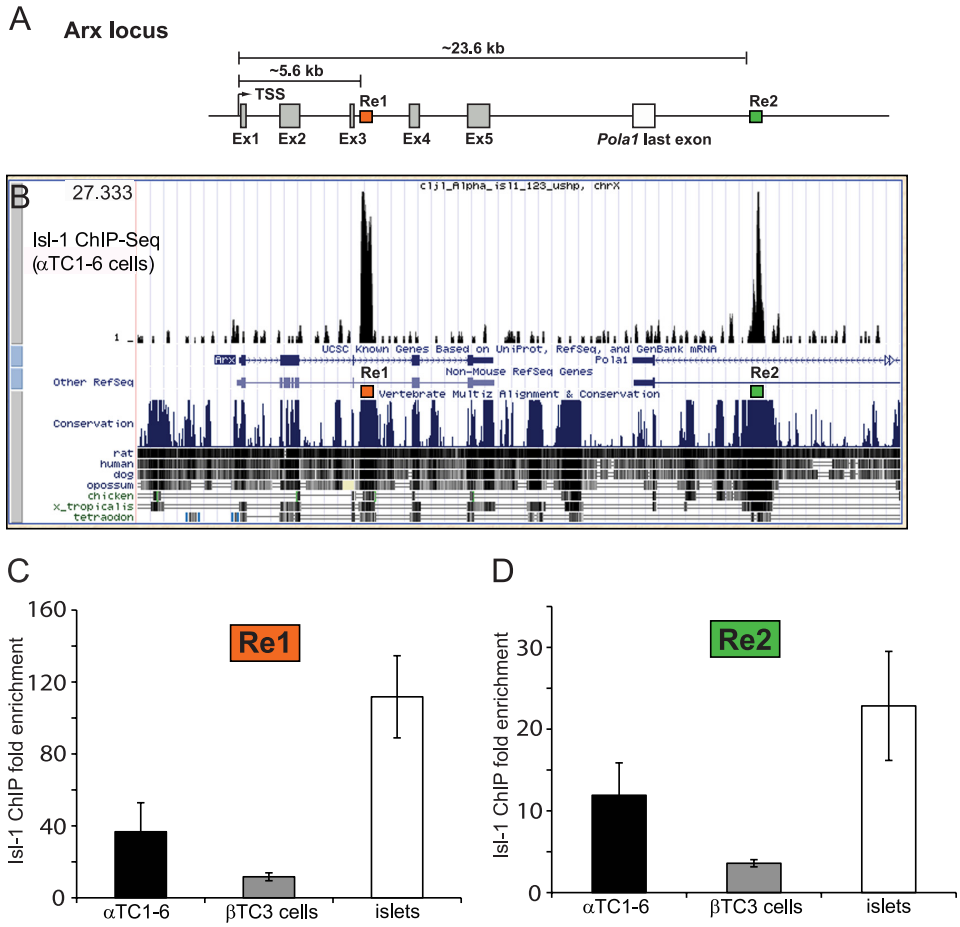

FIGURE 3. **Isl-1 binds to Re1 and Re2 of the mouse *Arx* gene.** *A*, schematic diagram of the *Arx* locus illustrating the locations of Re1 (*orange box*, +5.6 to +6.1) and Re2 (*green box*, +23.6 to +24 kb). *TSS*, transcription start site; *Ex*, exon. *B*, the ChIP-Seq image depicts Isl-1 occupancy within *Arx* Re1 (chrX:89544959–89545500) and Re2 (chrX:89563002–89563440) in αTC1–6 cells. The conservation between mouse and other species within this region was shown using the University of California Santa Cruz genome browser. *C* and *D*, Re1 and Re2 binding to Isl-1 was readily detected in adult mouse islets and αTC1–6 cells by standard ChIP but to a lesser extent in βTC3 cells. Results were normalized to Isl-1 binding to the *PEPCK* promoter. Data are presented as the mean ± S.E. $n = 3$; p value <0.05.

and not the insulin$^+$ β-cell population (Fig. 2, *I* and *J*). This *in vivo* result further suggested that Isl-1 stimulated *Arx* expression during α-cell development.

Isl-1 Binding Sequences Are Located in the Arx 3′-Noncoding Region—Isl-1 binding regions were identified in the *Arx* locus by whole-genome ChIP-Seq of α-Isl-1 antibody precipitates from nuclei of αTC1–6 cells, a transformed α-cell line (26). Binding was observed within two distinct conserved noncoding regions, one located ~5.6 kb downstream from the transcription start site within intron 3 (termed Region 1: Re1, +5.6 to +6.1 kb) and the other ~18.kb kb further away, within the last intron of *Pola1* (Region 2: Re2, +23.6 to +24 kb) (Fig. 3*A* and *B*).

Standard α-Isl-1 ChIP assays were performed over Re1 and Re2 in αTC1–6 cells, freshly isolated mouse islets, and βTC-3 (mouse insulinoma) cells to examine the specificity of binding (Fig. 3, *C* and *D*). These real-time PCR results were normalized to phosphoenolpyruvate carboxykinase promoter binding, an inactive gene in the pancreas. Isl-1 bound much more effectively to Re1 and Re2 in αTC1–6 than βTC-3 cells (Fig. 3, *C* and *D*). These data strongly suggested that the Isl-1 binding observed in islets is principally in α-cells and potentially important to *Arx* gene transcription.

Isl-1 Stimulated Re1- and Re2-directed Transcription in α-Cells—Re1, Re2, and Re1/Re2 combined were subcloned upstream of the minimal RNA polymerase II promoter in the p*GL4.27* luciferase reporter vector, and their activation properties were analyzed in transfected αTC1–6 cells. All of these p*GL4.27-Arx* constructs were more active than p*GL4.27* alone (Fig. 4*A*). Moreover, Isl-1 enhanced activation of each of the *Arx*-driven constructs in cotransfection assays, although p*GL4.27*-Re2 (2.9-fold) was more sensitive than either p*GL4.27*-Re1/2 (2.6-fold) or p*GL4.27*- Re1 (1.5-fold) (Fig. 4*A*).

To further investigate the involvement of Isl-1 in *Arx* activity, αTC1–6 cells were infected with an *Isl-1* shRNA expressing lentivirus. A nearly 70% reduction in cellular Isl-1 protein amount resulted in a 2-fold reduction in endogenous *Arx* mRNA levels (Fig. 4, *B* and *C*). Notably, Isl-1 knockdown also decreased p*GL4.27*-Re1 and p*GL4.27*-Re2 activity by ~2-fold in αTC1–6 cells (Fig. 4*D*), strongly suggesting that Isl-1 control of *Arx* transcription is mediated through Re1 and Re2.

Isl-1 Binding Mutants Reduced Re1- and Re2-mediated Stimulation in α-Cells—Several potential homeodomain-binding sites (TAAT/ATTA) were found within the highly conserved

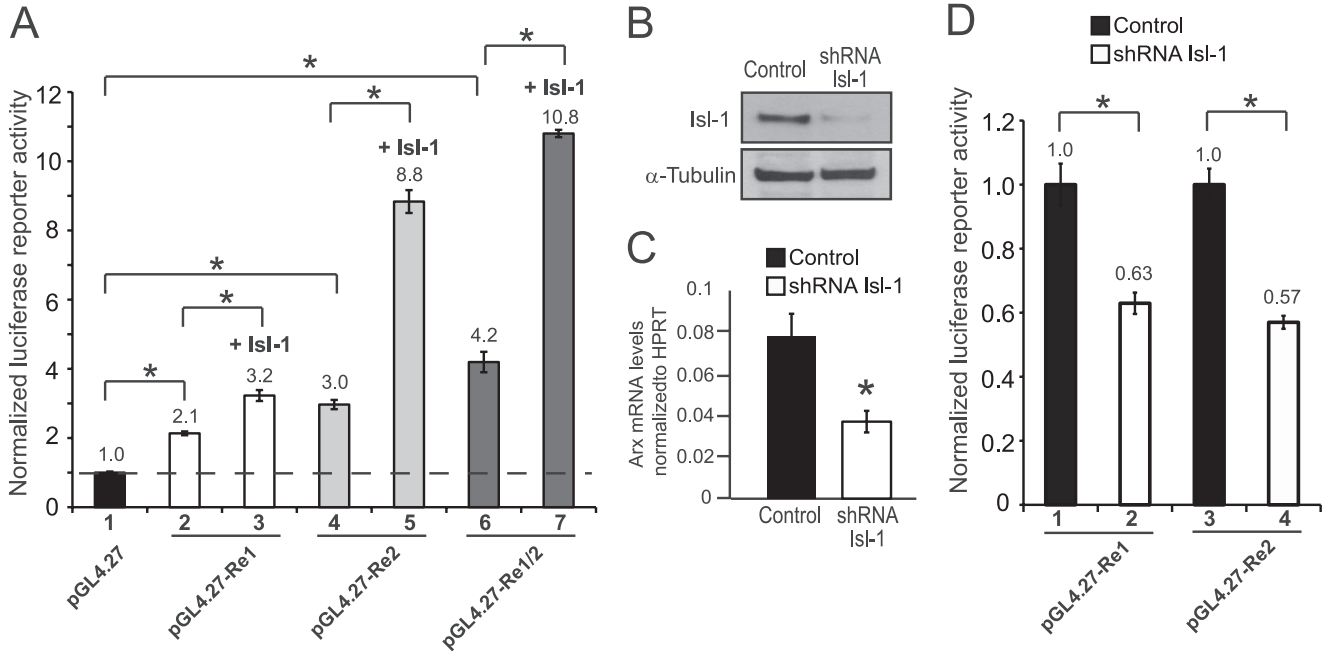

FIGURE 4. **Re1 and Re2 mediate *Arx* transcription in αTC1–6 cells.** *A*, p*GL4.27*-Re1, p*GL4.27*-Re2, and p*GL4.27*-Re1/2 were transfected into αTC1–6 cells in the presence or absence of a Isl-1-Myc expression plasmid. *B*, Western blot analysis using Isl-1 and control α-tubulin antibodies demonstrates a ~70% reduction of Isl-1 protein levels in *Isl-1* shRNA lentivirus-treated αTC1–6 cells. *C*, endogenous *Arx* mRNA expression was down-regulated relative to *HPRT* in Isl-1 shRNA treated αTC1–6 cells. *D*, Isl-1 shRNA knockdown reduced p*GL4.27*-Re1 and p*GL4.27*-Re2 reporter activity in αTC1–6 cells. Results are presented as the mean ± S.E. *, $p < 0.05$. p*GL4.27-Arx* activity was normalized to that of the p*RL-SV40 Renilla* luciferase in *A* and *D*.

sequences of Re1 and Re2 (Fig. 5, *A* and *B*). EMSAs were performed to evaluate the binding ability of *in vitro* translated Myc-tagged Isl-1 protein to six *Arx* sites from each region (Fig. 6, *A* and *B*), with the well characterized binding element from *MafA* Region 3 (*MafA*-R3) serving as the positive control (17). Isl-1 and Myc antibodies were used to locate the Isl-1-Myc complex in these assays. Isl-1-Myc:MafA was competed to varying degrees by all of the wild-type Re1 and Re2 sequences (Fig. 6, *A* and *B*), and their binding differences were also evident upon analyzing endogenous Isl-1 binding to the Re2–2, Re2–3, and Re2–6 probes (Fig. 6*C*). These results suggested that Isl-1 was able to bind to multiple sites within Re1 and Re2.

Isl-1 binding mutations in Re1–1, Re1–3, Re2–2, Re2–3, and Re2–6 (marked in *red*, Fig. 5, *A* and *B*) were generated in the p*GL4.27-Arx* reporters to evaluate their effect on activation in αTC1–6 cells (Fig. 5*C*). These sites were chosen on the basis of the strength of binding to Isl-1 (Fig. 6). The relatively weak binding mutant Re2–3 reduced p*GL4.27-Arx* activity to the same extent as the strong Re1–1, Re1–3, Re2–2, and Re2–6 (Fig. 5*C*). These results indicated that Isl-1 activation of *Arx* transcription in α-cells is likely through many related *cis*-elements within the 3′-flanking Re1 and Re2 control domains.

DISCUSSION

The results of this study reveal for the first time that Isl-1, an islet transcription factor, is essential for *Arx* transcription in α-cells. Although the importance of *Arx* during α-cell formation has been demonstrated in mice and humans (15, 16, 22, 23, 24), the molecular mechanisms involved in *Arx* transcription in the islet α-cell have remained largely elusive. Here, we have demonstrated that Isl-1 is an activator for *Arx* transcription in α-cells through direct binding to multiple elements in the 3′-noncoding sequences. We found that Isl-1 overexpression in islet cells leads to enhanced *Arx* expression in α-cells. These findings not only shed new light on the transcriptional regulation of *Arx* in α-cell biology, but also identify Isl-1 as an essential islet transcriptional activator for α-cell formation.

Isl-1 proteins have been shown to directly regulate gene activation (*e.g.* glucagon, insulin, and *MafA*) in islet cells by binding to the 5′-control regions of these genes (17, 31, 32). It is intriguing that Isl-1 binds to the *Arx* locus in two distinct regions that are located in the intron and 3′-noncoding sequences that are ~18 kb apart from each other. From our luciferase reporter assay, it is evident that these two regions have an additive effect when placed next to each other. Lin 1/Islet 1/Mec3-HD transcription factors, like Isl-1, have been shown to function in the context of coregulators that form a high-order transcriptional complex to exert their actions (33, 34). Whether coregulators like Lin 1/Islet 1/Mec3-domain binding proteins, Ldb1, or Ldb2, (33, 35) influence Isl-1 activation during pancreatic development remains to be investigated.

Interestingly, mice lacking ultraconserved elements (uc467) which encompass our Re2, have been generated by Ahituv *et al.* (36). Surprisingly, these mutant mice failed to reveal any overt abnormalities, although the specific impact on α-cell formation was not analyzed in their study (36). If no defect is observed, it is possible that Re1 might play a redundant role in mediating *Arx* transcription in developing islet α-cells. Outside of the pancreas, Arx expression is also detected in the developing ventral telencephalon of the brain, where its expression is directly regulated by the homeodomain factor Dlx2 (37, 38). Two Dlx2 binding sites (HD-1 and HD-2) have been identified in the

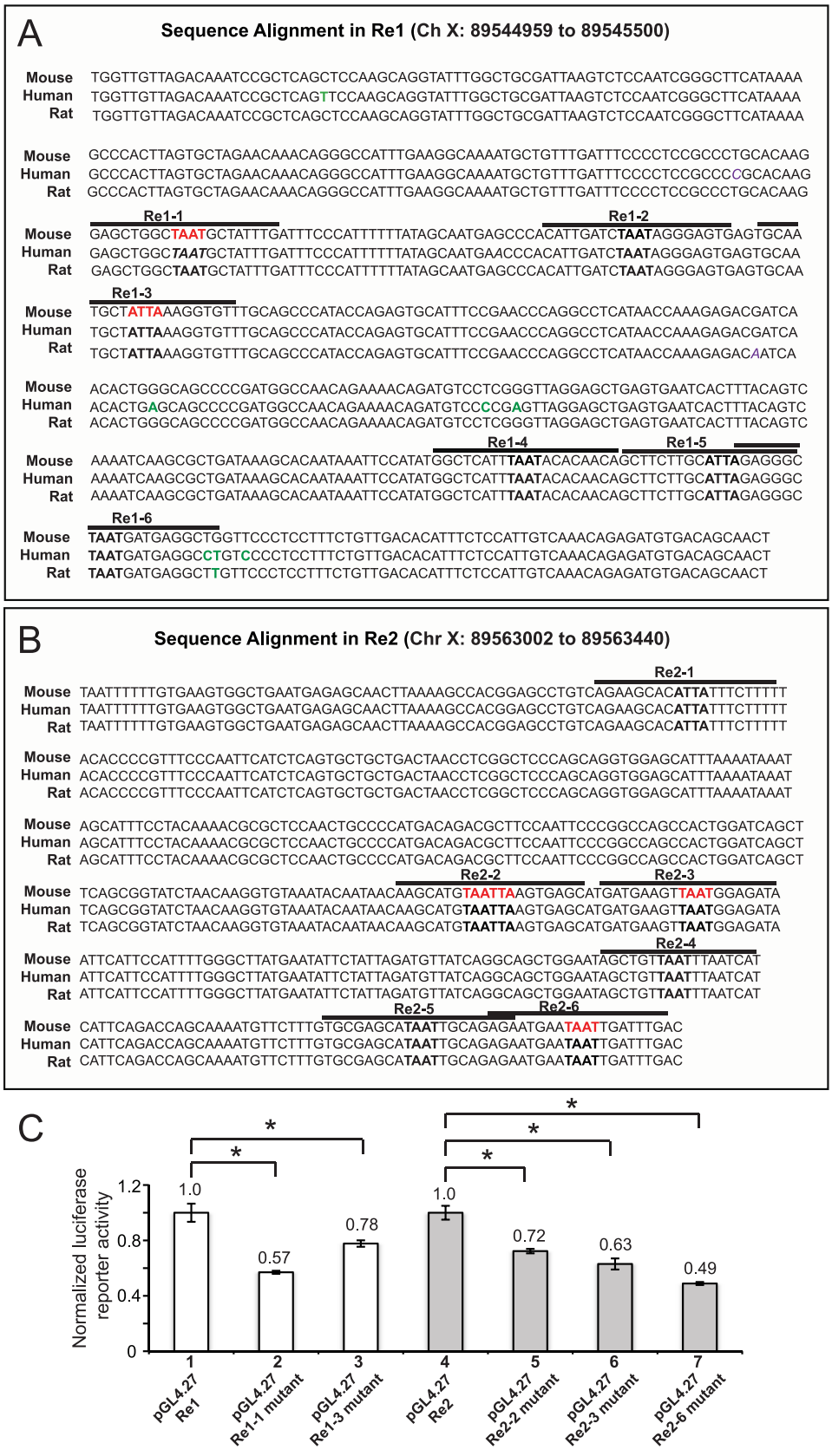

FIGURE 5. **The Isl-1 binding sites in Re1 and Re2 are necessary for αTC1–6 cell activity.** Sequence identity within *Arx* Re1 (*A*) and *Arx* Re2 (*B*) between mouse, human, and rat. Core Isl-1 binding site sequences are shown in *bold*, and the lines above demarcate the EMSA probe. Nucleotide mutated for luciferase reporter (*C*) and EMSA (Fig. 6*C*) assays are labeled in *red*. Nonconserved nucleotides are labeled in green. *C*, the activity of wild-type and Isl-1 binding site mutants (*red*) of p*GL4.27*-Re1 and p*GL4.27*-Re2 in αTC1–6 cells. The p*RL-SV40* normalized data were presented as the mean ± S.E. *, $p < 0.05$.

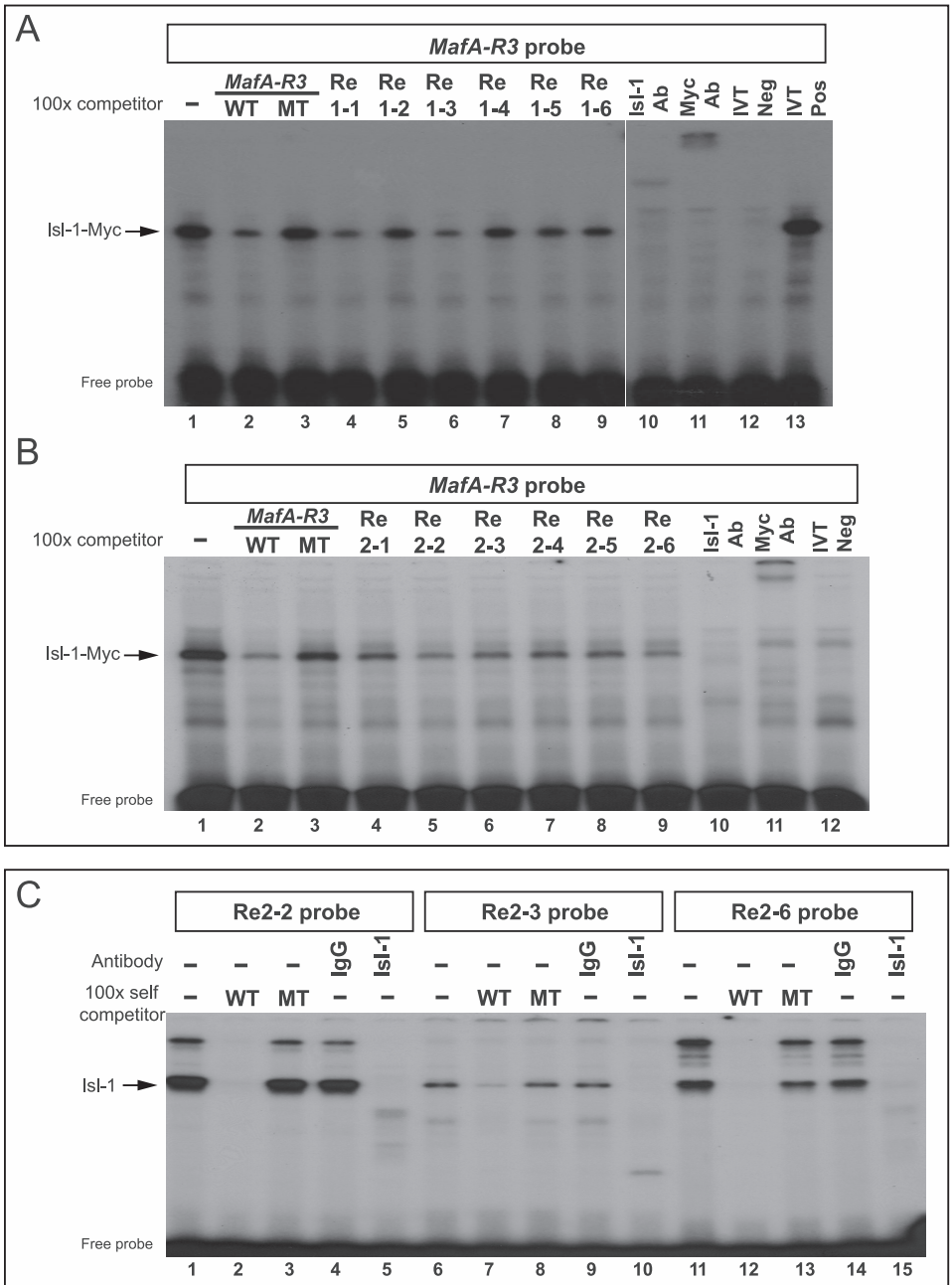

FIGURE 6. **Isl-1 binding sites in Re1 and Re2.** *A* and *B*, as a screen of putative Isl-1 binding elements, the radiolabeled *MafA-R3* Isl-1 site probe (17) was used in reactions with *in vitro* translated Isl-1-Myc and MafA-R3, Re1, or Re2 competitor oligonucleotides. Isl-1 and Myc-epitope antibodies were used to localize the Isl-1-Myc:MafA complex. *Ab*, antibody; *IVT*, *in vitro* translation; *Neg*, without Isl-1-Myc; *Pos*, with Isl-1-Myc. *C*, Re2–2, Re2–3, and Re2–6 probes were incubated with αTC1–6 nuclear extract. The specificity of Isl-1:Arx-Re2 binding was determined by Isl-1 antibody addition and competition with excess of unlabeled wild-type (*WT*) and Isl-1 binding site mutant (*MT*).

highly conserved noncoding sequence of the *Arx* locus (referred to as mUAS3, 37), which encompasses the Re2 sequences. HD-1 and HD-2 are the equivalent of Re2–2 and Re2–4 in our study. Notably, Dlx2 is minimally expressed as compared with Isl-1 in the pancreas (data not shown), suggesting that Isl-1 is the major transcriptional activator of *Arx* in developing α-cells.

Isl-1 has widespread effects in many developing tissues including motor neurons, cardiac mesoderm, and the pancreas (17, 39–41). Our findings reveal a novel role for Isl-1 in controlling the islet α-cell phenotype by directly regulating *Arx* transcription. Although *Arx* is detected in both α-cell progenitor and fully differentiated glucagon-producing cell populations (16), Isl-1 is only found in the latter population, which is post-mitotic and fully differentiated (39, 42, 43). Therefore, Isl-1 is only necessary for the maintenance of *Arx* transcription in forming α-cells and not involved during the initial actions of *Arx* in early α-cell specification. Finally, although our current study also suggests that Isl-1 is likely to regulate *Arx* transcription in adult islet α-cells, the *in vivo* role Isl-1 plays in adult islet *Arx* expression and α-cell biology await to be investigated.

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