Transcriptional Regulation of X-Box-binding Protein One (XBP1) by Hepatocyte Nuclear Factor 4α (HNF4A) Is Vital to Beta-cell Function*

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The transcription factor, X-box-binding protein-1 (XBP1), controls the development and maintenance of the endoplasmic reticulum (ER) in multiple secretory cell lineages. We show here that Hepatocyte Nuclear Factor 4α (HNF4α) directly induces XBP1 expression. Mutations in HNF4α cause Mature-Onset Diabetes of the Young 1 (MODY1), a subset of diabetes characterized by diminished GSIS. In mouse models, cell lines, and ex vivo islets, using dominant negative and human disease-allele point mutants or knock-out and knockdown models, we show that disruption of HNF4α caused decreased expression of XBP1 and reduced cellular ER networks. GSIS depends on ER Ca\(^{2+}\) signaling; we show that diminished XBP1 and/or HNF4α in β-cells led to impaired ER Ca\(^{2+}\) homeostasis. Restoring XBP1 expression was sufficient to completely rescue GSIS in HNF4α-deficient β-cells. Our findings uncover a transcriptional relationship between HNF4α and Xbp1 with potentially broader implications about MODY1 and the importance of transcription factor signaling in the regulation of secretion.

Cells use transcription factors to regulate expression of gene cohorts that coordinate response to stress, determine specific developmental fates, and scale intracellular architecture during development and disease(1, 2). When the biosynthetic load of a cell is increased and misfolded proteins accumulate in the endoplasmic reticulum (ER), the volume and composition of the ER is altered to facilitate the synthesis and processing of nascent polypeptides via the unfolded protein response (UPR) pathway. One of the principal components of the UPR is the transcription factor X-box-binding protein 1 (XBP1), which is canonically activated via IRE1 splicing of the XBPI transcript during ER stress (3, 4). However, XBP1 also establishes and maintains the subcellular machinery for synthesizing large quantities of protein during the normal development of professional secretory cells (2, 5). While the majority of genes activated by XBP1 are involved in ER biogenesis, up to 40% of its targets are not directly linked to the ER-stress response (6), further supporting its functions outside the UPR. In addition, the increased XBP1 that coordinates the scaling up of the secretory apparatus during development and homeostasis of dedicated secretory cells does not seem to require activation of the UPR (7). How XBP1 is induced and maintained during differentiation of secretory cells even in the absence of substantial ER stress is unclear, but a potential alternative mechanism is that Xbp1 may also be transcriptionally regulated. Hepatocyte Nuclear Factor 4-alpha (HNF4α) is a highly conserved transcription factor responsible for orchestrating the early development and maintenance of multiple adult organs. As a master developmental regulator, HNF4α likely acts upstream of the factors that establish the extensive cellular machinery required in professional secretory cell lineages within those organs. Despite overlapping expression and function, no direct relationship between HNF4α and Xbp1 has yet been described.

HNF4α is vital for β-cell function, and indeed, human mutations in HNF4α cause Mature-Onset Diabetes of the Young 1 (MODY1), a subset of diabetes characterized by diminished glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells, and susceptibility to type II diabetes (8, 9). While we know that β-cells require HNF4α to function, we understand little about the mechanistic/physiological role of HNF4α in these cells. Previous work showed that disrupting HNF4α expression in vivo in mouse islets resulted in diminished GSIS similar to that observed in MODY patients with HNF4α mutations (10, 11). Loss of HNF4α also was observed to disrupt Ca\(^{2+}\) signaling, though the mechanisms underlying those defects remain unclear. Decreased ER function is a plausible mechanism for the loss of function in MODY1 β-cells, because insulin secretion in β-cells is diminished if ER homeostasis is disturbed (12, 13).

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The abbreviations used are: ER, endoplasmic reticulum; XBP, X-box-binding protein; HNF, hepatocyte nuclear factor; MODY, Mature-Onset Diabetes of the Young; GSIS, glucose-stimulated insulin secretion; UPR, unfolded protein response.

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Defects in ER-related proteins contribute to multiple diabetic phenotypes in humans (14), and HNF4α is known to be important for maintaining ER stress response (15). In addition, knocking down XBP1 specifically in β-cells also leads to significantly reduced GSIS (16). Finally, disruption of calcium homeostasis in the ER also leads to impaired GSIS (17). Here we have characterized how XBP1 expression is governed at the transcriptional level and establish HNF4α as a direct transcriptional regulator of its expression. This implicates HNF4α in the maintenance and establishment of secretory cell ER networks. Accordingly, we report that both HNF4α and XBP1 are required to maintain ER calcium homeostasis and GSIS in β-cells. In addition, we show that restoration of XBP1 expression alone in islets deficient for HNF4α is sufficient to rescue impaired GSIS. Thus, the results may provide new insight toward discerning why dysfunction in HNF4α causes the pathophysiological findings in MODY1 patients.

**Experimental Procedures**

*Cell Lines and Transient Transfection—* Min6 cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose, supplemented with 10% fetal calf serum, 2 mM l-glutamine, 25 mM Heps, and 285 μM 2-mercaptoethanol, and penicillin and streptomycin. INS-1 832/13 cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), penicillin, and streptomycin, sodium pyruvate and β-mercaptoethanol. Human embryonic kidney (HEK)-293 cells (ATCC) were cultured in DMEM containing 10% FBS and penicillin and streptomycin. INS-1 cells containing doxycycline inducible dnHNF4α were treated with 500 ng/ml doxycycline to induce expression as previously described(36). All cells were passaged at 90% confluence using trypsin-EDTA. For overexpression of HNF4α in INS-1 cells coding regions of human HNF4α (obtained from Addgene) were subcloned into a pcDNA3.1 expression vector, and 5 μg of each plasmid or the pmaxGFP (Lonza) control plasmid were precipitated by either preimmune control or HNF4α (obtained from Addgene) or whole rabbit serum (preimmune control) antibody to assess the quantity of genomic sequences immunoprecipitated (ChIP) experiment. Ten microliters of anti-HNF4α (Santa Cruz Biotechnology), mouse antimyc-tag (Cell Signaling), and rabbit anti-α- and β-tubulin (Cell Signaling). Secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse Ig (Santa Cruz Biotechnology).

*Chromatin Immunoprecipitation—* Chromatin immunoprecipitation (ChIP) was performed as described previously(47). A 5, 6–8-week-old WT mice were homogenized and used for this ChIP experiment. Ten microliters of anti-HNF4α (Santa Cruz Biotechnology) or whole rabbit serum (preimmune control) together with protein A/G plus-agarose (Santa Cruz Biotechnology) was added to the homogenized tissue for immunoprecipitation. Quantitative real-time PCR (qRT-PCR) was performed (primer sequences are available from corresponding author) to assess the quantity of genomic sequences immunoprecipitated by either preimmune control or HNF4α antisum, as well as a 1:10 dilution of the cell extract prior to immunoprecipitation (input). The two predicted HNF4α binding sites described above were probed in addition to an intrinsic control region with no predicted HNF4α binding sites nearby. Data are graphed as a percentage of precipitated DNA:total input (genomic DNA).

*Beta-cell Morphological Characterization using Immunofluorescence—* Pancreata were prepared and stained as described previously(49). Briefly, they were fixed with freshly prepared formalin and suspended in fixative for 24 h at room temperature, followed by multiple rinses in 70% ethyl alcohol (EtOH), arrangement in 2% agar in a tissue cassette, and routine paraffin processing. Sections (5 μm) were deparaffinized and rehydrated, and then antigen retrieval was performed by boiling in 50 mM Tris–HCl, pH 9.0. Slides were blocked in 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in phosphate-buffered saline (PBS) and then incubated in goat anti-Calregulin (Santa Cruz Biotechnology) followed by AlexaFluor594 anti-goat. Fluorescence microscopy and imaging were performed using a Zeiss Axiovert 200 microscope with Axiocam MRM camera and Apotome II instrument for grid-based optical sectioning.

For morphological analysis, three 5-μm sections taken 100-μm apart were stained with hematoxylin and eosin to allow identification of islets. Whole slides were scanned with the Nanozoom microscope and the cross-sectional area of islet/
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total pancreas tissue was measured across each slide using Nanozoom Digital Pathology and software (Hamamatsu). Samples were randomized, and the scorer was blinded to ensure unbiased quantification. Values are expressed as %β-cell area.

**Mouse Studies**—All experiments involving animals were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Floxed Hnf4α, CAGGCreERTM transgenic mice were generated by crossing Hnf4αfloxed/+ mice (a gift from Frank Gonzalez, NIH)(23), with CAGGCreERTM;Hnf4αfloxed/+ (48) mice to allow systemic, tamoxifen-inducible knock out of HNF4α. 6–8-week-old CAGGCreERTM;Hnf4αfloxed/+ mice and CAGGCreERTM;Hnf4αfloxed/+ littermate controls were injected intraperitoneally with tamoxifen (5 mg/20g body weight, 5 consecutive days) to induce Cre-mediated deletion. Mice were sacrificed 4 weeks after first tamoxifen injection. No control siRNA to knockdown HNF4α was blind relative to condition. Nuclear areas were identified by Hoescht staining, and pixels with an intensity of >30 gray value as determined as previously described(51), and the ratio values were plotted against time.

**Islet Isolation and Culture**—Pancreatic islets from CAGGCreERTM;Hnf4αfloxed/+ mice and CAGGCreERTM; Hnf4αfloxed/+ littermate controls were isolated as previously described (52), by pancreatic duct injection of 1000 U/ml of collagenase solution (Sigma) followed by digestion at 37 °C for 15 min with mild shaking. Islets were washed several times with Hanks balanced salt solution, separated from acinar cells by straining through a 100-μm filter, viewed under a dissecting microscope, and handpicked for culture (yield = 200–300 islets/mouse). Isolated Islets were maintained in RPMI1640 supplemented with 10% FBS and penicillin and streptomycin at 37° with 5% CO2. All islets were allowed to recover from isolation for 24 h before analysis. Islets were isolated from mice in random order relative to condition.

**Adenoviral Transduction**—Unspliced XBP1 adenovirus (Applied Biological Materials), LacZ Adenovirus (Applied Biological Materials), and spliced XBP1 (a gift from Laurie Glomcher) (53), were amplified in HEK293 cells, cultured as described above. Infected cells were lysed by three cycles of freezing and thawing and then centrifuged. Viral titer was determined by infecting HEK-293 cells with serially diluted viral stock and overlaying with agar and subsequently counting the resulting plaques. INS-1 cells containing dnHNF4α cells were treated with doxycycline or vehicle to induce expression of dnHNF4α as described above. Five days post-treatment, cells were infected with either XBP1 or LacZ adenovirus at a mul-
Multiplicity of infection (MOI) of 100. Viral stock was replaced with complete medium after 2 h of infection. Isolated murine islets were infected as described previously(54). Briefly, 70 islets/condition were washed in cold PBS, pretreated with HBSS containing 2 mM EGTA at 37° with 5% CO₂ for 15 min, then infected with adenovirus in serum-free RPMI 1640. Following a 15-min incubation, complete medium was added to islet culture. Islets were infected for 24 h before GSIS assay and harvesting RNA. Adenovirus was used in isolated islets at the following MOIs: LacZ MOI = 50, XBP1u MOI = 50, XBP1s MOI = 10.

Glucose-stimulated Insulin Secretion Measurement—For INS-1 GSIS assay, 2 days post- adenoviral infection cells were washed with PBS, then incubated for 1 h in Krebs-Ringer Buffer containing 3 mM glucose. After 1 h, cells were washed with PBS, and basal insulin secretion was measured by incubating cells for 1 h in Krebs-Ringer Buffer containing 3 mM glucose. Medium was sampled, then replaced with medium containing 16.7 mM glucose, or 200 μM Tolbutamide for 1 h. Medium was collected and analyzed for insulin content by ELISA using the SINGulex Erenna platform by the Washington University Diabetes Research Center Immmuomassay Core. Static GSIS was similarly measured in isolated islets as previously described(55). 24 h post-infection, fifty islets were placed in Krebs-Ringer Buffer containing 3 mM glucose to measure basal insulin secretion, then stimulated with 16 mM glucose. Insulin secretion was measured in each condition as described above.

Graphing and Statistics—All graph values represent the mean of the sample, and error bars represent S.E. where indicated. Significance was determined using Student’s t test or ANOVA with Dunnet’s posthoc comparison as indicated. Wherever possible, samples were randomized and measurements were blinded to prevent the introduction of experimental bias. Sample sizes were determined based on statistical significance and practicality.

Results

Regulation of Xbp1 by HNF4α—To elucidate the potential transcriptional regulation of XBP1, we identified evolutionarily conserved binding sites in the human XBP1 promoter by aligning regions of synteny, then screening them with the Transfac transcription factor binding site database(18). Two regions with high conservation containing putative HNF4α binding sites, 1.4 and 2.6 kilobases upstream of the Xbp1 transcription start site, were identified using first the Transfac transcription factor binding library and then affirmed using a previously published algorithm developed to search for sites of high HNF4α binding affinity (19). These putative binding sites were constitutively occupied by HNF4α in mouse pancreatic, measured via chromatin immunoprecipitation (Fig. 1A). Overexpression and knockdown experiments in vitro showed HNF4α was both sufficient and necessary for normal Xbp1 expression in pancreatic β-cell derived-cell lines. Disrupting HNF4α either by siRNA knockdown (Fig. 1, B and C) or by overexpressing a doxycycline-inducible dominant-negative version of HNF4α that lacks a DNA-binding domain and has been shown to bind to endogenous HNF4α (Fig. 1, D and E) (36), resulted in a 65–75% decrease in Xbp1 expression in INS-1 and MIN-6 cells. Conversely, overexpression of HNF4α via transient transfection caused a 5-fold increase in Xbp1 expression (Fig. 1F). To further substantiate this transcriptional relationship, we analyzed the effects of Hnf4a deletion on Xbp1 expression in other tissues by mining published microarray studies and by direct qRT-PCR analysis of adult (Fig. 1G) and embryonic liver (20) as well as adult small intestine (data not shown but available on Gene Expression Omnibus under accession numbers GSE34581, GSE3124, GSE3126) (21–23). Again, the results showed a consistent trend toward correlation of Xbp1 expression decrease with loss of Hnf4a in multiple secretory tissues.

Various single point mutations in the HNF4α locus have been identified in human patients afflicted with impaired β-cell function. To better understand the impact of these mutations, we designed two HNF4α expression vectors, each containing one of the most prevalent mutations (24, 25). Overexpression of each of these individual mutants in INS-1 cells resulted in a ~4-fold decrease in the expression of XBP1 mRNA (Fig. 1H). Interestingly, although we could detect the transfection-mediated increase in mRNA for both mutant and wild-type HNF4α qPCR, we could detect only wild-type HNF4α protein by Western blot (Fig. 4D and data not shown). We speculate the mutant genes may encode protein products that are abnormally folded and/or truncated and degraded. In any case, all the data support the conclusion that common HNF4α mutations found in human patients decrease abundance of Xbp1.

HNF4α Is Required for ER Maintenance in Vivo—The mechanisms whereby disruptions of HNF4α in MODY1 cause β-cell dysfunction remain an area of open debate. As XBP1 is critical in scaling up and maintaining the ER of professional secretory cells, we hypothesized mutations in HNF4α may impair β-cell function via dysregulation of Xbp1 and consequent ER dysfunction. Previous studies of effects of loss of Hnf4a in islets focused on deletion of the gene during development. As we were concerned here with the effects of adult onset of aberrant Hnf4a on islet function, we induced deletion of Hnf4a<sup>flo</sup>/<sup>flo</sup> mice using a global, tamoxifen-inducible Cre in 6–8-week-old mice. As predicted, deleting Hnf4a in existing islets resulted in significant loss (~60%) of both Xbp1 expression in islets and, in turn, of XBP1 transcriptional targets like Edem1 (Fig. 2A) when compared with littermate Hnf4a<sup>flo</sup>/<sup>+</sup> controls (referred to hereafter as “WT”) (26). Supporting previous findings, insulin and Hnf1α mRNA levels were unaffected by loss of Hnf4a (10, 11). While XBP1 establishes secretory cell machinery and maintains cell architecture (2, 27), likely in large part independent of activation of the UPR (28), in chronic/long-term ER stress conditions, XBP1 is a fundamental component of the UPR (29). We sought to characterize the ER-stress state of ΔHNF4α β-cells to determine whether other branches of the UPR were activated to compensate for loss of Xbp1. One of the most reliable methods of measuring UPR activation in cells is by quantifying the expression of genes whose transcription is enhanced by activated master regulators of the UPR (30). Decreased Xbp1 in Hnf4a<sup>Δ/Δ</sup> mice did not cause increase in such UPR transcripts like Chop, Bip, or Atf4, nor a decrease in mRNA levels of the ER-marking Calregulin (CRP55) (Fig. 2B). However, loss of XBP1 following deletion of Hnf4a did correlate with a nearly 7-fold reduction in ER area in β-cells (Fig. 2, C and D). Despite decreased ER in each cell, overall islet mass, as measured by
microscopic area through cross-sectioned pancreata, was not changed in *Hnf4αΔ/Δ* pancreata (Fig. 2E), indicating that *Hnf4α* is not required to maintain islet number or size. Thus, loss of *Hnf4α* caused diminished ER network, a phenotype similar to that caused by deleting *Xbp1* from existing adult secretory cells (2, 5).

**Hnf4α and Xbp1 Are Necessary to Maintain ER Calcium Homeostasis**—In previous reports, constitutive deletion of *Hnf4α* from islets early in development, as opposed to in the adult, caused impaired GSIS(10, 11). The mechanism of decreased GSIS was hypothesized to be dysregulated cytoplasmic Ca<sup>2+</sup> signaling in response to glucose, but the molecular mechanism driving this impairment has remained unclear. Ca<sup>2+</sup> signaling depends on the ATP-dependent closure of KATP channels, triggering membrane depolarization and opening voltage-gated Ca<sup>2+</sup> channels(31). Thus, one mechanism that could mediate how loss of *Hnf4α* could cause GSIS could be via disruption of those channels. However the sulfonylurea Tolbutamide, which closes KATP channels leading to membrane depolarization, stimulated less insulin secretion in INS-1 cells expressing dominant negative-HNF4α (dnHNF4α, described above) than in normal INS-1 cells (Fig. 3). Additionally, qPCR analysis showed abundance of transcripts for the KATP channel subunits Sur1 (Abbc8) and Kir6.2 (Kcnj11) in *Hnf4αΔ/Δ* islets were unchanged relative to littermate controls (Fig. 4A). These data, along with previous work showing impaired insulin secretion even upon depolarization with KCl (10), suggest the defect in the glucose response pathway due to disrupted HNF4α is distal.
to KATP channel dependent-membrane depolarization and that it depends, in part, on loss of XBP1. Though KATP channel expression was unchanged in Hnf4α islets, expression of the putative XBP1 transcriptional target, Serca2b(Atp2a2), was significantly reduced (Fig. 4A) (32). SERCA2b is an ER Ca\(^{2+}\) pump, responsible for establishing and maintaining the large calcium gradient between the ER and cytoplasm (33). Intracellular stores of Ca\(^{2+}\) are critical for GSIS, because Ca\(^{2+}\) release from these stores triggers secretion of insulin granules. Accordingly, decreasing ER Ca\(^{2+}\) stores and/or flux has been shown to disrupt GSIS (17). Thus, in Hnf4α\(^{Δ/Δ}\) mice, the decreased expression of a key molecular driver of the ER Ca\(^{2+}\) gradient suggested that disruption of ER Ca\(^{2+}\) stores may play a critical role in the GSIS abnormalities seen in the absence of normal HNF4α. We used an ER-specific FRET sensor to measure ER [Ca\(^{2+}\)] in ΔHnf4α β-cells (12, 34). The D1ER cameleon construct encodes two fluorophores conjugated to a calmodulin molecule that is targeted specifically to the ER lumen with a KDEL sequence. When it binds Ca\(^{2+}\), the cameleon undergoes a conformational change that approximates the fluoros to produce FRET activity quantitatively proportional to ER [Ca\(^{2+}\)] (Fig. 4B). Changes in FRET:No FRET ratios correspond with changes in ER [Ca\(^{2+}\)] as observed when treating cells permeabilized by ionomycin with either CaCl\(_2\) to increase ER [Ca\(^{2+}\)] or EGTA/thapsigargin to diminish it (Fig. 4C). In accordance with the mechanism of lost GSIS in MODY1 being disruption of XBP1-mediated ER Ca\(^{2+}\) stores, both knockdown of HNF4α and pharmacological inhibition of XBP1 activation (using 4μ8C, which blocks IRE1-mediated conversion of the XBP1 transcript to the active spliced form) in INS-1 cells resulted in decreased ER [Ca\(^{2+}\)] (Fig. 4C) and loss of HNF4α or XBP1 protein levels as expected (Fig. 4D). Simultaneously knocking down HNF4α and pharmacologically inhibiting XBP1 did not augment the decreased ER [Ca\(^{2+}\)]. Thus, it is likely that HNF4α and XBP1 work via the same pathway to maintain high ER [Ca\(^{2+}\)] in adult β-cells. To further explore the requirement of HNF4α for proper Ca\(^{2+}\) signaling in β-cells, we observed changes in cytoplasmic Ca\(^{2+}\) levels in response to various stimuli in INS-1 cells transiently transfected with scrambled siRNA or siRNA targeting HNF4α using Fura 2AM-based Ca\(^{2+}\) imaging. Expectedly, ΔHnf4α β-cells exhibited a diminished response to stimulation with 16.7 mM glucose, as observed in
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FIGURE 3. Tolbutamide-induced insulin secretion. Doxycycline-induced expression of the dominant negative form of HNF4α from Fig. 1 (DE) in INS-1 cells completely abrogated increased insulin secretion in response to tolbutamide exposure, suggesting the MODY1 secretion defect is distal to β-cell membrane depolarization in the GSIS signaling cascade. Infection with an adenovirus carrying Xbp1u expression vector is sufficient to rescue this GSIS defect. (Means ± S.E. of n = 3 biological replicates depicted, statistical significance by one-tailed Student’s t test.)

other MODY1 β-cell models (Fig. 4E). To identify the cause of this deficit in Ca²⁺ signaling, and further explore our previous results indicating HNF4α is required for ER Ca²⁺ homeostasis, we exposed these cells to 20 mM Caffeine. Caffeine is an agonist of the ryanodine receptor that stimulates release of Ca²⁺ from stores in the ER and thus an increase in cytoplasmic [Ca²⁺] (35). Caffeine induced diminished cytoplasmic [Ca²⁺] increase in ΔHnf4α β-cells (Fig. 4F), indicating that ER Ca²⁺ homeostasis is disrupted. In short, loss of ER Ca²⁺ in ΔHnf4α β-cells may underlie their impaired GSIS.

XBP1 Is Sufficient to Rescue Insulin Secretion in ΔHnf4α β-cells—We next sought to confirm the physiological relevance of the HNF4α → XBP1 relationship by determining if we could rescue aberrant GSIS in the absence of HNF4α simply by restoring XBP1. If XBP1 were decreased in the absence of HNF4α due to direct loss of transcriptional up-regulation by HNF4α, as we hypothesized, then restoration specifically of the unspliced XBP1 (Xbp1u) form should rescue GSIS, because Xbp1u is the unmodified mRNA directly generated from transcription of the XBP1 gene (i.e., prior to activation by IRE1-mediated splicing). Restoring the deficit of Xbp1u transcript caused by loss of HNF4α would allow cells the freedom to use the normal IRE1 splicing mechanism to produce only as much active Xbp1s transcript as they require for ER homeostasis. We returned to our in vitro model employing transgenic INS-1 cells containing a doxycycline inducible dnHNF4α described above (Figs. 1 and 3) (36). As shown above, the functional loss of HNF4α activity in insulin-secreting cells when doxycycline is added to induce dnHNF4α, caused both decreased XBP1 (Fig. 1, D and E) and loss of GSIS (Fig. 5A). This decreased GSIS was completely rescued by adenoviral transduction of Xbp1u. As discussed above, forced expression of Xbp1u was also sufficient to rescue impaired insulin secretion in response to sulfonylurea treatment in these dnHNF4α β-cells (Fig. 3). We repeated this study ex vivo, using Hnf4α−/− islets isolated 3 weeks following tamoxifen-induced Hnf4α−/− deletion. As expected, cultured islets had impaired GSIS due to HNF4α deficiency. Xbp1u restoration increased the direct XBP1 transcriptional targets Edem1 and Serca2b (Fig. 5B) confirming that transduction of Xbp1u restored functional XBP1-mediated transcriptional activity to scale up expression of its normal transcriptional targets. Remarkably, as predicted, restoring unspliced Xbp1 expression in these ex vivo cultured, HNF4α-deficient β-cells was also sufficient to completely rescue their GSIS, indicating that the impaired GSIS in the absence of HNF4α depends on transcriptional maintenance of XBP1 expression by HNF4α (Fig. 5C). Because of the direct transcriptional regulation of XBP1 by HNF4α and the lack of GSIS enhancement in WT β-cells transduced with Xbp1u, the ability of Xbp1u to rescue the phenotype caused by loss of HNF4α is likely because it corrects the diminished basal Xbp1 expression in ΔHnf4α β-cells.

We also transduced spliced XBP1 (Xbp1s) in isolated ΔHNF4α mouse islets, bypassing the normal regulation of transcriptionally regulated Xbp1u by IRE1α splicing. Transduction of Xbp1s rescued the XBP1 targets, Edem1 and Serca2b (Fig. 6A) but resulted in GSIS roughly 50% lower than that in control WT islets (Fig. 6B). That result is consistent with previous reports that β-cell homeostasis is compromised by forced expression of spliced Xbp1 because cells must be able to dynamically regulate XBP1 levels via the endogenous IRE1 splicing mechanism, with direct forced expression of high levels of already activated Xbp1 potentially being toxic (37). Accordingly, Hnf4α−/− islets infected with Xbp1s exhibited GSIS rescue to the levels observed in WT islets infected with Xbp1u, suggesting that, while forced expression of Xbp1s is detrimental to β-cell health, it is still able to compensate for GSIS defects in β-cells caused by the absence of HNF4α.

Discussion

We report that Xbp1 is a direct transcriptional target of HNF4α in multiple secretory tissues. Given the importance of HNF4α mutations in diabetes, we have focused on the relationship between HNF4α and XBP1 specifically in insulin-secreting β-cells. Deletion of HNF4α in β-cells causes them to lose XBP1, which in turn causes dismantling of ER. HNF4α point mutants designed to match mutations that cause human diabetes also resulted in loss of XBP1 in vitro. Loss of either HNF4α or XBP1 leads to diminished ER Ca²⁺, which in turn impairs GSIS, a defect that can be completely rescued by reestablishing normal Xbp1 transcript levels (Fig. 5D). Together, our results identify a new transcriptional relationship between evolutionarily-conserved genes, Xbp1 and Hnf4αΔΔ, involved in fundamental development and disease in multiple tissues.

We also demonstrate specific cellular contexts during which Xbp1 expression is functionally regulated at the transcriptional level. XBP1 is induced in response to accumulation of unfolded protein in the ER by splicing of its message via the endonuclease IRE1α, and the canonical view of how XBP1 abundance is modulated concern that mechanism. However, the transcriptional regulation of Xbp1 may be as important to its role in secretory cell development as its activation by IRE1. Xbp1 expression is significantly induced and maintained at much higher levels in
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secretory cells, and large pools of unspliced Xbp1 mRNA are required to restore homeostasis in chronic ER stress conditions (38). Additionally, IRE1 is basally activated in dedicated secretory cells at levels comparable to those observed during acute ER stress in non-secretory cells (>40%) (39). When the presumably UPR-activating biosynthetic load-stimulus is removed in B-lymphocytes differentiating to plasma cells by disrupting expression of IgM, XBP1 transcript levels still increase, indicating that XBP1 activation is differentiation-dependent rather than UPR-dependent in certain secretory cells (40). Together, these studies suggest that transcription of Xbp1 is the rate-limiting step in its activation during secretory cell development and maintenance.

It is somewhat surprising that HNF4α, which is largely studied in developmental contexts as a master regulator of differentiation in endodermal organs, is required for continued maintenance of XBP1 in differentiated, adult cells. However, unbiased, comprehensive screens for genes whose expression depends on HNF4α have previously identified XBP1 as a potential target (14-17), and chromatin immunoprecipitation fol-
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FIGURE 5. XBP1 is sufficient for GSIS in ΔHnf4α β-cells. A, glucose-stimulated insulin secretion (GSIS) was determined by harvesting supernatant from wildtype INS-1 and doxycycline-inducible dnHNF4α INS-1 cells following incubation for one hour under high (16 mM) glucose conditions. Induction of dominant negative HNF4α abrogates GSIS (insulin secretion of −1 means no induction relative to baseline insulin secretion with 3 mM glucose). All cells were transduced by adenovirus carrying either unspliced Xbp1 or LacZ control vectors. Note that Xbp1 transduction rescues GSIS in dnHNF4α cells (means ± S.E. of n = 6 experiments depicted, statistical significance by one-tailed Student’s t-test). B, ΔHNF4α or heterozygote control islets were cultured for 24 h after isolation and then transduced with either LacZ or Xbp1 vector-containing adenovirus. 24 h later, RNA was harvested and qRT-PCR performed for transcripts from Hnf4α, Xbp1 and two downstream transcriptional targets of XBP1, Serca2b, and Edem1 (data represent means ± S.E. from three individual islet isolations and transduction experiments). C, normalized GSIS was determined as for panel A with transduction of either LacZ or Xbp1 vector-containing adenovirus into isolated islets. Note isolated islets from Hnf4α−/− mice exhibit a complete lack of GSIS. (Data represent means ± S.E. from three individual islet isolations and transduction experiments). D, model of MODY1 pathology. XBP1 regulates transcription of multiple genes like SERCA2B that induce and maintain normal ER and ER Ca2+ function. Loss of HNF4α in patients with MODY1 causes reduced XBP1 expression.

Followed by sequencing (ChIP-Seq) has also shown peaks indicating potential binding of HNF4α to the putative XBP1 promoter (41). Thus, though the results of the previous screens have not been validated and the direct relationship between HNF4α and XBP1 has apparently never been specifically studied prior to the current work, our results are not entirely unprecedented. Indeed, HNF4α has also been shown to regulate expression of ankyrin repeat and sterile α motif domain containing 4b (Anks4b), a protein that binds ER chaperones and augments the ER stress response, supporting our hypothesis that HNF4α is required for the establishment and maintenance of the ER in β-cells (42).

In ongoing work in our lab, we also observe that Xbp1 expression depends on HNF4α in the stomach, and, exactly as in β-cells, loss of HNF4α in mature gastric chief cells causes dismantling of the ER.3 The results are consistent with work by us and others showing that continued XBP1 is required for ER in gastric chief cells(2) and in β-cells (16). On the other hand, though we observe Xbp1 expression clearly depends on HNF4α

3 B. D. Moore and J. C. Mills, unpublished observations.

in the liver (see Fig. 1G plus previous studies available in GEO that we have analyzed (21–23)), we have not been able to detect substantial effects on ER in that organ when HNF4α is deleted from mature hepatocytes (data not shown). Actually, this is in agreement with previous studies showing that direct depletion of XBP1 itself from mature hepatocytes is not sufficient to cause ER dismantling in existing cells (53). Together, the results suggest that there are tissue-specific differences in the role of XBP1 in regulating the ER: perhaps XBP1 is generally required for establishment of an elaborate ER network in dedicated secretory cells but is only required in certain cell types for its continued, homeostatic maintenance.

While previous studies suggest alterations in the KATP channel may play a role in MODY1 pathology (10, 11) they also show that MODY1 β-cells have impaired GSIS even upon membrane depolarization with KCL, suggesting the cause of the diminished GSIS is distal to the KATP channel/membrane depolarization event. ER Ca2+ homeostasis is important for myriad cellular processes and plays a pivotal role in intracellular Ca2+ signaling. Our data indicate that XBP1 and HNF4α are required for maintaining this homeostasis. Though inflamma-
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**FIGURE 6. Rescue of GSIS in ΔHNF4α islets with spliced Xbp1.** A, ΔHNF4α and heterozygote control islets were isolated and treated as in (Fig. 5B) but infected with adenovirus harboring plasmid a transcript for spliced XBP1 (XBP1s). XBP1s expression restores mRNA abundance of downstream targets, Edem and Serca2b, to normal levels in ΔHNF4α islets, but GSIS (i.e. insulin release following switching of islets to 16 mm glucose) is diminished in both WT and ΔHNF4α islets upon transducing expression of XBP1s when compared with WT GSIS. LacZ vector-containing adenovirus was used as a control for transduction (means ± S.E. of n = 3 experiments depicted, statistical significance was determined by one-tailed Student’s t test).

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