Activation of FoxM1 revitalizes the replicative potential of aged β-cells in male mice and enhances insulin secretion

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Abstract

Type 2 diabetes incidence increases with age, while β-cell replication declines. The transcription factor FoxM1 is required for β-cell replication in various situations, and its expression declines with age. We hypothesized that increased FoxM1 activity in aged β-cells would rejuvenate proliferation. Induction of an activated form of FoxM1 was sufficient to increase β-cell mass and proliferation in twelve-month-old male mice after just two weeks. Unexpectedly, at two months of age, induction of activated FoxM1 in male mice improved glucose homeostasis with unchanged β-cell mass; cells expressing activated FoxM1 demonstrated enhanced glucose-stimulated Ca$^{2+}$ influx, which resulted in improved glucose tolerance through enhanced β-cell function. Conversely, our lab has previously demonstrated that mice lacking FoxM1 in the pancreas display glucose intolerance or diabetes with only a 60% reduction in β-cell mass, suggesting that the loss of FoxM1 is detrimental to β-cell function. *Ex vivo* insulin secretion was therefore examined in size-matched islets from young mice lacking FoxM1 in β-cells. *Foxm1*-deficient islets indeed displayed reduced insulin secretion. Our studies reveal that activated FoxM1 increases β-cell replication while simultaneously enhancing insulin secretion and improving glucose homeostasis, making FoxM1 an attractive therapeutic target for diabetes.
INTRODUCTION

Diabetes afflicts 347 million people worldwide (1) and is associated with reduced functional β-cell mass (2; 3). In the United States, the incidence of diabetes increases from less than 5% in people between 20-44 years of age to more than 25% in people above 65 (4). The increased incidence of diabetes with age inversely correlates with β-cell proliferation (5-7), the primary mechanism for increasing β-cell mass after early postnatal life (5; 8).

Mouse models confirm that β-cell replication decreases with age (9; 10). In mice as well as in humans, proliferation is highest prior to sexual maturity and continues to decline throughout adulthood. In addition to a decline in baseline replication, the ability of β-cells to respond to proliferative stimuli also diminishes with age. In mice at two months of age, pregnancy, streptozotocin treatment, high-fat diet, treatment with the glucagon-like peptide 1 receptor analog exendin-4, and partial pancreatectomy all potently increase β-cell replication (9; 11). By eight months of age, however, only partial pancreatectomy can stimulate β-cell proliferation, and by 14 months of age, none of these stimuli is able to increase β-cell replication over baseline. A goal for treating Type 2 diabetes (T2D), therefore, is to induce replication in endogenous β-cells. However, dividing β-cells often display sub-optimal performance (12), so simply increasing proliferation may not result in increased functional β-cell mass. Ideal therapies for diabetes would both expand β-cell mass and enhance insulin secretion.

A partial explanation for the diminution in β-cell proliferation with age is an accumulation of the cell cycle inhibitor p16\textsuperscript{Ink4a}, encoded by Cdkn2a, in aged β-cells (10). Overexpression of p16 in young, non-stimulated mice decreases baseline β-cell proliferation to that observed in much older mice but has no effect on β-cell division in older mice; conversely,
loss of p16 increases proliferation in aged β-cells to levels observed in young mice but has no effect on β-cell proliferation in young mice. The transcription factor forkhead box M1 (FoxM1) indirectly negatively regulates Cdkn2a through activation of c-Myc and Bmi1 (13; 14). FoxM1 is required for β-cell proliferation in several situations, including postnatal growth, pregnancy, and partial pancreatectomy (15-17). Deletion of Foxm1 in the pancreas manifests post-weaning as a 60% deficit in β-cell mass accompanied by diabetes or glucose intolerance in male mice (15).

Although full-length FoxM1 is required for β-cell proliferation, it is not sufficient to promote β-cell proliferation in young mice, even in response to the replicative stimulus of 60% partial pancreatectomy (17). The inability of full-length FoxM1 to promote β-cell division likely results from post-translational regulation of FoxM1 activity. Previous work suggests that transduction of human islets by full-length FOXM1 can increase β-cell replication. However, this work was performed ex vivo and β-cell replication may have been affected by growth factors in the media that are not present in vivo (18). We therefore employed a mouse model we derived in which an activated form of FoxM1 lacking its N-terminal intramolecular repressor domain can be induced specifically in β-cells by doxycycline (Dox) treatment (referred to as β-FoxM1* mice) (19). After 2 weeks of activated FoxM1 expression in aged mice, β-cell mass and proliferation as well as glucose homeostasis was examined. Our results demonstrate that activated FoxM1 can counteract the age-related decline in β-cell replication and highlight an unappreciated role for FoxM1 in enhancing insulin secretion. Altogether, these experiments suggest FoxM1 as a novel therapeutic target for simultaneously enhancing β-cell mass and function to treat diabetes.
RESEARCH DESIGN AND METHODS

Mice. RIP-rtTA (20), HA-TetO-FoxM1^{ANRD} (19), RIP-Cre (21), and Foxm1^{fl} (22) mice have been described previously. RIP-rtTA mice were maintained on a C57Bl6/J background, HA-TetO-FoxM1^{ANRD10} and HA-TetO-FoxM1^{ANRD14} on a C57Bl6/JxDBA mixed background, RIP-Cre and Foxm1^{fl} mice were maintained on a mixed C57Bl6/JxDBAx129Sve background, and Foxm1^{LCA} mice were maintained on a mixed C57Bl6/Jx129Sve background. Mice were housed in a controlled-temperature environment with a 12-hour light/dark cycle. All experiments were performed on male mice except when examining P8 mice, when both sexes were used, and for Foxm1 and target gene expression analysis, when female C57Bl/6J mice were used. Experimental mice or dams were administered water containing 2% Dox supplemented with sucralose (two weeks for experimental mice and from E9.5 to P8 for dams). All procedures were approved and performed in accordance with the Vanderbilt Institutional Animal Care and Use Committee.

The Foxm1^{LCA} allele was generated using BAC recombineering, which is described in detail in (23). Briefly, ~500 bp regions of homology ~6Kb upstream and ~11Kb downstream from the Foxm1 transcriptional start site (TSS) (regions “A” and “D” in Supplementary Fig. 1A) were amplified by PCR from the bacterial artificial chromosome (BAC) bMQ-387I22 ( geneservice) and cloned into the HindIII and NotI sites of pBS-DTA using standard procedures. PmeI and SwaI sites were added in the NotI site. This new plasmid with regions of Foxm1 homology was recombined using EL350 cells into to bMQ-387I22 ( geneservice) to replace exons 2-4 with a selection cassette encoding puΔTK and Neomycin (Supplementary Fig 1A). ~500 bp sequences of Foxm1 1.3 Kb upstream and 8 Kb downstream of the Foxm1 TSS (regions “B” and “C” in Supplementary Fig 1A) were cloned into pLCA.71.2272NTK+XhoI. This vector
was used to retrieve the modified Foxm1 sequence through recombination in EL350 cells. The resulting plasmid was then linearized with Swal and electroporated into 129Sve ES cells, which were positively selected with neomycin and negatively selected with gancyclovir. Electroporation and antibiotic selection were performed by the Vanderbilt Transgenic/ES Cell Shared Resource.

Surviving cells were screened by Southern blot analysis after digesting ES cell DNA with Xhol and probing with a fragment of outside of the 5’ region of homology (indicated by bar 5’ of “A” in Supplementary Fig 1A). Untargeted clones yielded a 20 Kb fragment while correctly targeted clones yielded a 9 Kb fragment. Positive clones were re-screened with the same digest/probe combination and also by digesting with FspI and probing for sequences outside the 3’ region of homology (probe represented by bar 3’of “D” in Supplementary Fig 1A), which yielded fragments of 23 Kb (WT) and 12 Kb (targeted). Southern blot probes were made by cloning ~500 bp regions of Foxm1 into pCR2.1 using the TOPO-TA cloning kit (Invitrogen) and excising them with EcoRI. Southern blotting was performed as previously described (24).

Out of 114 colonies, three (2, 5, and 6 in Supplementary Fig. 1B) displayed the correct banding pattern at both the 5’ and 3’ ends. Clone 6 with 40% normal karyotype was selected to expand and inject into blastocysts. Two 90% male chimeras achieved germline transmission. Mice were genotyped using PCR with the following primers: 5’-CTA GTG GTT GAT CTC ACC AGT; 5’-TGT GGC ATG TGG GTC AAT CCT; 5’-TAC CGG TGG ATG TGG AAT GTG. All other primer sequences are available upon request.

**Immunolabeling and morphometry.** Tissue was processed as previously described (25). β-cell mass measurements were performed as previously described, on slides through the entire
pancreas 250 µm apart, resulting in 6-12 slides per mouse. (25). Slides were imaged with a Scanscope CS or FL (Leica Biosystems), or for H2AX immunolabeling, with a LSM 710 META Inverted confocal microscope (Carl Zeiss). Cell counts were performed manually on 3 sections 500 µm apart as previously described (19); cell counts per mouse per experiment ranged from at least 5000 to >75,000. Cell size was calculated as previously described (19). Islet size distribution was measured by circling islets on sections used for β-cell mass assessment within ImageScope (Leica Biosystems). ImageScope software automatically displays areas of islets. Proliferation assessments within islets sorted by size was performed manually and normalized to the total β-cell area for the islets within the bin size, since large islets comprised much more total area than small islets.

Antibodies used: guinea pig anti-insulin (1:500-1:1000; Dako), mouse anti-p16 (1:50-1:100; Santa Cruz Antibodies), mouse anti-HA (1:50-1:100; Cell Signaling), rabbit anti-γH2AX (1:500; Cell Signaling), rabbit anti-FoxM1 (1:500-1:1000; Santa Cruz), rabbit anti-Ki67 (1:500; BDBioscience), Alexa 488 conjugated-mouse anti-HA (1:50; Cell Signaling), HRP-conjugated donkey anti-guinea pig (1:400; Jackson Laboratories), Alexa 488-conjugated donkey anti-guinea pig (1:400; Jackson), Alexa 647-conjugated donkey anti-guinea pig (1:400; Jackson), Alexa 555-conjugated anti-rabbit (1:400; Jackson), and Alexa 555-conjugated anti-mouse (1:400; Jackson). Immunohistochemistry and immunofluorescence were performed as previously described (19) with the following exceptions: 1) HA, Ki67, and p16 immunolabeling required citric acid buffer antigen retrieval; 2) for HA immunolabeling, overnight incubation occurred at RT; 3) since p16 immunolabeling was oversaturated with incubation at RT, p16 and HA incubation occurred sequentially rather than in parallel. For p16/HA double labeling, mouse-anti-p16 labeled with a Zenon mouse Ig2a Alexa 555 kit (Life Technologies) and a direct-Alexa 488-labeled mouse-anti-
HA antibody (1:50, Santa Cruz) were used. To examine embryonic heart and liver structure as well as to non-quantitatively assess DNA content, sections were labeled with 0.5 µg/mL DAPI for one minute.

**Quantitative Real-Time PCR.** RNA extractions and qPCR were performed as previously described (19).

**Cytoplasmic Calcium Measurements.** Islets from β-FoxM1*14 mice were dispersed with 0.05% Trypsin after incubation overnight in RPMI containing 15% FBS and 2µg/mL Dox. Dispersed islets were incubated (20 minutes, 37°C) in Krebs-Ringer buffer (KRB) supplemented with 2 μmol/mL fura-2 acetoxymethyl ester (Molecular Probes). Fluorescence imaging was performed using a Nikon Eclipse TE2000-U microscope equipped with an epifluorescence illuminator (Sutter, Inc), a CCD camera (HQ2; Photometrics Inc), and Nikon Elements software. Cells were perifused at 37°C at a 2 mL/min with KRB solutions that contained 2 mM (first three minutes) or 14 mM glucose (next 22 minutes). The ratios of emitted fluorescence intensities at excitation wavelengths of 340 and 380 nm (F340/F380) were determined every 5 seconds. Ca^{2+} imaging was followed by immunolabeling for insulin and the HA-tag on activated FoxM1; after imaging, single cells that expressed activated FoxM1 were compared to those that did not.

**Metabolic assays.** Intraperitoneal glucose tolerance tests (IPGTT) were performed as previously described (26). For insulin during IPGTT measurements, blood was collected from the saphenous vein; insulin content was measured by the Vanderbilt Hormone Assay and Analytical Core. For insulin tolerance tests, mice were fasted for six hours, blood glucose was measured,
and mice were injected with 0.8 U insulin/kg body weight. Blood glucose was measured periodically for 90 minutes.

**Statistics.** All data are plotted as mean ± SEM. One-way ANOVA, two-way ANOVA, two-way ANOVA with repeated measures, with Tukey or Bonferroni post-tests, or Student’s t-test were used to analyze data, as appropriate. One-way ANOVA was used to assess liver and heart size while the Chi-Square test was used to evaluate. p-values <0.05 were considered significant
RESULTS

Foxm1 and target expression decline with age. Since FoxM1 indirectly negatively regulates p16 (13; 14), and p16 accumulates with age, we predicted that expression of Foxm1 and its direct targets would decline with age. We therefore examined expression of Foxm1, S-phase associated kinase 2 (Skp2), and Aurora B kinase (Aurbk) in islets from two-, four-, eight-, and twelve-month-old mice. A significant decline in Foxm1 was apparent as early as four months of age. By twelve months of age, all three proliferation-associated genes were significantly downregulated (Fig. 1).

β-cell mass and proliferation increase in aged mice expressing activated FoxM1. We hypothesized that restoring FoxM1 activity in aged β-cells would rejuvenate their replicative potential. Control (RIP-rtTA) and β-FoxM1* embryos were exposed to Dox to induce expression of activated FoxM1 beginning at embryonic day (E) 9.5, just after the pancreas starts to form. β-FoxM1* mice exhibited no change in β-cell mass or ad libitum-fed blood glucose at postnatal day 8 (P8; Fig. 2A-D). Similarly, when eight-week-old β-FoxM1* mice were treated with Dox for two weeks, there was no change in β-cell mass or proliferation compared to controls (Fig. 2E-H).

However, at four, eight, and twelve months of age, β-cell mass was increased in β-FoxM1* mice after only two weeks of activated FoxM1 induction (Fig. 3A, D-E). Because of the marked increase in β-cell mass and the known recalcitrance of β-cells to proliferative stimuli at twelve months of age, we focused our attention on this time-point for further study.

β-cell proliferation was also increased at twelve months of age after two weeks of FoxM1 induction (Fig. 3B, F-G). While a trend towards increased β-cell proliferation was apparent in
islets of all sizes, robust and significant increases in β-cell proliferation were apparent only in islets smaller than 1000 µm (Fig. 3H). No significant difference in islet size (Suppl. Fig. 2) or the number of individual β-cells located near ducts (0.022 ± 0.003 for RIP-rTA mice and 0.029 ± 0.012 in β-FoxM1* mice) was observed between the two genotypes. A trend toward increased numbers of islets in the smallest and largest bins, however, may explain the overall increase in β-cell mass in β-FoxM1* mice. The lack of difference in β-cell size in β-FoxM1* mice (Fig. 3I) supports β-cell proliferation as the mechanism by which β-cell mass increased in β-FoxM1* mice.

Our previous studies revealed that activated FoxM1 can be detected in about ten percent of β-cells at any given time, yet robust proliferation and β-mass expansion was observed. These results suggested that FoxM1 activation might result in non-cell-autonomous effects on β-cell proliferation. Expression of activated FoxM1 was examined in proliferating and non-proliferating β-cells (Fig. 3C, J-K). A higher percentage of FoxM1*-expressing cells replicate than cells lacking activated FoxM1, indicating that FoxM1 is acting in a cell-autonomous manner, consistent with its known role in transactivating cell-cycle genes (14; 22; 27). The lower overall replication in Fig. 3C compared to Fig. 3B results from the different labeling conditions used.

Activated FoxM1 bypasses p16 to re-activate the cell cycle in aged β-cells. Since FoxM1 inhibits expression of Cdkn2a, p16 expression was examined in aged β-FoxM1* mice. As has been reported previously (11; 28), p16 could be observed in nearly all β-cells at twelve months of age in control mice (data not shown), and this was also true in β-FoxM1* mice, even in β-cells expressing activated FoxM1 (Fig. 4A-B), indicating that increased β-cell replication in aged
β-FoxM1* mice does not result from decreased p16. Another marker of cell cycle arrest, phosphorylated histone H2AX (γH2AX) (29) was, however, significantly downregulated in β-FoxM1* mice (Fig. 4C-E). Since γH2AX is also a marker of DNA damage, we assayed for TUNEL+ β-cells but did not observe any in control or β-FoxM1* mice (n=6, 3 slides per mouse), indicating that activated FoxM1 may bypass p16 to overcome cell cycle arrest and activate cell cycle progression.

**FoxM1 regulates β-cell function.** Increased β-cell mass may not be sufficient to improve insulin secretion or glucose homeostasis since increased β-cell replication is sometimes associated with decreased β-cell function (12). We therefore examined glucose homeostasis in β-FoxM1* mice. At two months of age, before any change in β-cell mass, β-FoxM1* mice exhibited improved glucose tolerance after receiving Dox, while no change was observed in RIP-rtTA mice (Fig. 5A-B). *Ad libitum*-fed blood glucose was normal (Fig. 5C). No difference was observed in insulin secretion during a glucose tolerance test (data not shown). No difference was observed in insulin tolerance between β-FoxM1* mice and RIP-rtTA mice (Fig. 5D), implying that the difference in glucose tolerance is a result of enhanced β-cell function. The improvement in glucose tolerance was still observed in twelve-month old β-FoxM1* mice (Fig. 5E-F) with no change in *ad libitum*-fed blood glucose (Fig. 5G).

To determine whether the effect on β-cell function was due to a neomorphic trait of activated FoxM1 or an endogenous role of FoxM1, perifusions were performed on size-matched islets from RIP-Cre mice, mice lacking FoxM1 in β-cells (*RIP-Cre;Foxm1fl/fl* mice, referred to as *Foxm1Δβ* mice), and mice lacking one copy of Foxm1 in the β-cell (*RIP-Cre;Foxm1+/fl* mice, referred to as *Foxm1+/−* mice). RIP-Cre mice were used as controls since they display impaired
glucose-stimulated insulin secretion (GSIS) compared to mice with no Cre expression (data not shown); a deleterious effect of the RIP-Cre transgene on β-cell function has been reported previously (30; 31). Size-matched islets are necessary since mice lacking Foxm1 have decreased β-cell mass (15). Islets from Foxm1Δβ mice exhibited impaired GSIS compared to both RIP-Cre and Foxm1Δβ/ mice (Fig. 5H), indicating that FoxM1 has an endogenous role in regulating β-cell function. Size-matched islets from Foxm1Δβ mice had normal insulin content (data not shown), suggesting that the defect in β-cell function lies downstream of insulin production.

To further determine where in the GSIS pathway FoxM1 exerts its regulatory effects and if FoxM1 is acting cell-autonomously, glucose-stimulated Ca\(^{2+}\) entry into dispersed β-cells from β-FoxM1* mice (19) was assessed. When glucose enters the β-cell, metabolic action causes an increased ATP/ADP ratio; this leads to closure of the ATP-dependent K\(^+\) channel and depolarization of the β-cell membrane potential, allowing increased Ca\(^{2+}\) influx. Ca\(^{2+}\) influx into β-cells causes insulin granules to fuse to the plasma membrane and release their insulin (32). Interestingly, cells expressing activated FoxM1 (HA\(^+\) cells) displayed increased Ca\(^{2+}\) influx during the time corresponding to the second phase of insulin secretion compared to cells not expressing activated FoxM1 (HA\(^-\) cells) (Fig. 6A: average traces; Fig. 6B: representative individual traces; and Fig. 6C: area under the curve for indicated time-points), indicating that the effect of insulin secretion is cell-autonomous. Observing this effect at the level of Ca\(^{2+}\) influx indicates that the enhancement of β-cell function achieved by increased FoxM1 activity occurs upstream of Ca\(^{2+}\) influx.

**FoxM1 is expressed at lower levels in quiescent and proliferating islet cells.** In most cell types, FoxM1 protein expression can only be detected in proliferating cells (33) and is rapidly degraded
after cytokinesis (34). Our data suggest that FoxM1 plays an important role in non-proliferating β-cells to promote β-cell function. To characterize the FoxM1 expression pattern in highly proliferative islets, we immunolabeled pancreas sections from Lep\textsuperscript{ob/ob} mice, which show β-cell hyperplasia in response to leptin deficiency and insulin resistance (35). We tested the specificity of the FoxM1 antibody on Foxm1\textsuperscript{LCA/LCA} embryos (Fig. 7A-D), a Foxm1 null mouse we recently generated (Suppl. Fig. 2). Foxm1\textsuperscript{LCA/LCA} mice recapitulate the phenotype of Foxm1\textsuperscript{−/−} mice (36): no Foxm1\textsuperscript{LCA/LCA} mice survive to birth (0 of 25; Chi-Squared=8.360; p-value=0.0153), and the number of Foxm1\textsuperscript{LCA/LCA} mice is reduced at E15.5 (7 of 53; Chi-Squared=3.931; p value=0.0474). Foxm1\textsuperscript{LCA/LCA} embryos display increased numbers of cells with endoreplication in both heart and liver at E15.5 (Suppl. Fig. 3), and liver weight is significantly reduced (Suppl. Fig. 4).

While FoxM1 was nuclear in the developing heart (A), it appeared cytoplasmic in the embryonic day (E) 15.5 pancreas; sequestration of FoxM1 in the cytoplasm has been reported as a way of controlling FoxM1 activity (37). Importantly, neither nuclear nor cytoplasmic reactivity was observed in heart (B) or pancreas (D) from Foxm1 null embryos.

Unlike many other cell types (33; 34), we found that all endocrine cells express FoxM1 in adult Lep\textsuperscript{ob/ob} mice (Fig. 7E-H). Most endocrine cells expressed low levels of FoxM1 (FoxM1\textsuperscript{LO} cells), but a few showed higher expression (FoxM1\textsuperscript{HI} cells; Fig. 7E). FoxM1\textsuperscript{HI} endocrine cells exhibited similar FoxM1 levels as the rare FoxM1\textsuperscript{+} exocrine cells (Fig. 7F). All of the FoxM1\textsuperscript{HI} endocrine cells expressed the proliferation marker Ki67 (Fig. 7G), as did all FoxM1-expressing acinar cells (Fig. 7H). In addition, RNA-Seq of sorted quiescent and cycling β-cells using CyclinB1-GFP revealed low levels of Foxm1 expression in non-proliferating β-cells compared with cycling β-cells (19 Fpkm in quiescent cells versus 1532 Fpkm in proliferative cells; personal communication from Dr. Yuval Dor, Hebrew University, Jerusalem). Thus, FoxM1\textsuperscript{LO}
cells appear to be non-proliferative, suggesting that lower levels of FoxM1 are sufficient to promote β-cell function, while higher levels of FoxM1 are associated with proliferation.
DISCUSSION

We demonstrate that expression of activated FoxM1 in aged β-cells triggers cell cycle progression, leading to elevated proliferation and increased β-cell mass. The ability to increase β-cell replication in aged islets is particularly important because aged β-cells are recalcitrant to replicative stimuli and because T2D onset increases with age in humans.

Previous successful efforts to increase β-cell replication in aged islets have included elimination or indirect downregulation of p16 in mouse islets (10; 28), or downregulation of the cell cycle inhibitor p57 in human islets (38). Considering that all successful endeavors to increase β-cell mass in aged mice have involved decreasing p16 and that FoxM1 negatively regulates p16 expression in other cell types, we expected p16 to be decreased in aged β-cells expressing activated FoxM1 and displaying increased β-cell mass and proliferation. Contrary to these expectations, no change in p16 was observed in activated-FoxM1-expressing cells. However, the senescence and DNA damage marker γH2AX was decreased in β-FoxM1* mice without any change in TUNEL+ cells, indicating that activated FoxM1 is bypassing p16 to permit aged β-cells to re-enter the cell cycle. Alternatively, the majority of β-cell replication, and thus the decrease in p16, could have occurred soon after activated FoxM1 induction rather than at the two-week time-point examined here. At twelve months, the expansion in β-cell mass two weeks after FoxM1 induction is substantial in the absence of any increase in ad libitum-fed blood glucose, suggesting that these mice are probably not insulin-resistant and that elevated blood glucose is not responsible for the increased β-cell proliferation. Compared to the increase in β-cell mass, the elevation in β-cell replication in twelve-month old β-FoxM1* mice is less impressive. This smaller-than-expected elevation in β-cell proliferation may reflect negative
feedback inhibition on the cell cycle by the two-week time-point examined; β-cell proliferation may have been much higher just after initiating activated FoxM1 expression.

At two months of age, before any elevation in β-cell mass, β-FoxM1* mice display improved glucose homeostasis, suggesting that FoxM1 regulates insulin secretion. We further showed that activated FoxM1 is acting cell-autonomously upstream of Ca^{2+} influx and downstream of insulin production to enhance insulin secretion.

In retrospect, a role for FoxM1 in β-cell function should not have been surprising since previous work from our lab demonstrated that male mice lacking FoxM1 in the pancreas displayed diabetes or glucose intolerance with only a 60% decrease in β-cell mass (15) and that mice expressing activated FoxM1 display improved blood glucose in response to STZ treatment before any improvement in β-cell mass (19). In addition, many mouse models can support a decrease in β-cell mass of 80% or more and still maintain glucose homeostasis (39). Furthermore, maintenance of euglycemia with a severe reduction in β-cell mass echoes what is observed in the progression towards Type 1 diabetes: patients display the most severe symptoms only after a loss in β-cell mass >90% (40). We therefore examined insulin secretion in size-matched islets from mice lacking FoxM1 specifically in β-cells and demonstrated that these islets secreted less insulin than control islets despite normal insulin content, confirming a previously unappreciated role for FoxM1 in the regulation of β-cell function. No increase in circulating insulin was observed during a glucose tolerance test in mice expressing activated FoxM1, however, which may indicate a faster disposal of insulin in peripheral tissues.

In pancreatic acinar cells, FoxM1 can only be detected within proliferating cells, but while FoxM1 is expressed at high levels in proliferating islet cells, it is also expressed at lower levels in all other islet cells. We propose a model in which FoxM1 is required for β-cell function,
activating genes involved in this process at low protein levels, but in order to surpass the threshold for cell cycle entry, FoxM1 must be expressed at higher levels (Fig. 7G). Taken together, our data indicate that changes in glucose homeostasis in β-FoxM1* mice are at least partially decoupled from increases in β-cell mass. Future studies will investigate how FoxM1 distinguishes its proliferative versus functional targets in the β-cell.

Our experiments indicate that FoxM1 acts cell-autonomously to enhance both β-cell proliferation and function, but we reported previously that only ~10% of β-cells express activated FoxM1 in β-FoxM1* mice (19). How, then, can gene expression changes in such a low proportion of β-cells translate to such a large change in β-cell mass in just two weeks? RIP-rtTA generally drives expression of its targets in a large percentage of β-cells. Unlike many proteins, however, endogenous expression of FoxM1 is dynamically regulated with the cell cycle, and although the HA-FoxM1ANRD transgene lacks a domain that directs FoxM1 to the proteasome, FoxM1 degradation may have additional layers of control. Activated FoxM1 may be expressed in only 10% of β-cells at any given time-point, but the pool of β-cells in which activated FoxM1 is expressed may be greater over time.

Much attention in the field of β-cell proliferation has focused on extracellular factors since they might be easier to modulate than intracellular factors. However, recent work has suggested that human β-cells are less responsive to extracellular growth factors than mouse β-cells because of reduced expression of receptors for these growth factors, especially with age (41-43). We show that activation of FoxM1 in aged β-cells can bypass inherent brakes present in these cells while enhancing function. Successful drugs inhibiting FOXM1 are on the market today (44), demonstrating the feasibility of targeting FOXM1 activity in vivo and suggesting that FoxM1 is a worthwhile therapeutic target for T2D.
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FIGURE LEGENDS

Figure 1. qRT-PCR reveals that expression of Foxm1 and its direct targets declines with age. (* p-value < 0.05 versus two-month islets; ** p-value < 0.01 versus two-month islets).

Figure 2. β-cell mass in young mice does not increase in response to increased FoxM1 activity. (A-B) Insulin labeling and Eosin staining on P8 RIP-rtTA (A) and β-FoxM1* (B) pancreas sections. (C) Quantification of P8 β-cell mass. (D) Ad libitum-fed glucose in P8 mice. (E-F) Insulin labeling and Eosin staining on two-month old RIP-rtTA (E) and β-FoxM1* (F) pancreas sections. (G-H) Quantification of β-cell mass (G) and proliferation (H) in two-month old mice.

Figure 3. β-cell mass and proliferation increase in aged mice expressing activated FoxM1. (A) β-cell mass in aging mice. (B) β-cell proliferation in twelve-month mice. (C) Quantification of Ki67 in activated FoxM1-expressing (HA⁺) or activated FoxM1-absent (HA⁻) β-cells at twelve months of age. (D-G) Representative images for data quantified in (A-B). Insulin labeling and Eosin staining (D-E), insulin and Ki67 labeling (F-G) on 12-month-old RIP-rtTA (D, F) and β-FoxM1* (E, G) pancreas sections. Arrows indicate positive cells. Images in F and G were acquired at 200X. Inset in (G) shows a higher resolution Ki67⁺ β-cell. (H) β-cell size in twelve-month-old mice. (I) Proliferation in size-sorted islets. (J-K) Representative image of data quantified in (C) with all three colors (J) or with only insulin and HA immunolabels (K). (*p<0.05).
Figure 4. FoxM1 decreases γH2AX with no change in p16. (A) Insulin, HA, and p16 labeling on 12-month-old and β-FoxM1* pancreas sections. (B) Single channel labeling of p16 from image in (A). Black arrows indicate β-cells positive for both p16 and activated FoxM1; images acquired at 200X. (C-D) Insulin and γH2AX labeling on RIP-rtTA (C) and β-FoxM1* (D) pancreas sections. White arrow indicates cell positive for both insulin and γH2AX. (E) Quantification of γH2AX+ β-cells. (Scale bar: 20 µm; *p<0.05)

Figure 5. FoxM1 regulates β-cell function. (A-B) IP-GTT on 2-month old RIP-rtTA and β-FoxM1* mice before and after two weeks of Dox administration. (*p<0.05, n=7-10). (C) Ad libitum-fed glucose in two-month old mice. (D) Insulin tolerance test in two-month-old RIP-rtTA and β-FoxM1* mice after two weeks of Dox treatment (n=4-6). (E-F) IP-GTT on twelve-month old RIP-rtTA and β-FoxM1* mice before and after two weeks of Dox administration. (*p<0.05, n=6-7). (G) Ad libitum-fed glucose in twelve-month old mice. (H) Perifusion of isolated islets from nine-week-old RIP-Cre, Foxm1+/−, and Foxm1−/− mice. (*, p<0.05 for Foxm1−/− mice versus RIP-Cre mice; †, p<0.05 for Foxm1−/− mice versus Foxm1+/− mice; ††, p<0.5 for Foxm1+/− mice versus RIP-Cre mice; G5.6 = glucose concentration of 5.6 mM, G16.7 = glucose concentration of 16.7 mM).

Figure 6. FoxM1 acts cell autonomously to enhance calcium influx upstream of insulin secretion. (A) Average calcium measurements from β-FoxM1*14 β-cells from mice expressing or lacking activated FoxM1. Dispersed β-cells were maintained in 2 mM glucose until 180 seconds, when they were treated with 14 mM glucose. (B) Representative calcium traces from
individual cells. (C) Area under the curve for HA\(^+\) and HA\(^-\) negative cells for indicated time period. (n=2 mice, 24-98 cells; *p<0.0005).

**Figure 7. FoxM1 is more highly expressed in proliferating \(\beta\)-cells.** (A-D) FoxM1 immunohistochemistry on E15.5 wild-type (A,C) and Foxm1-null (B,D) hearts (A,B) and pancreas (C,D). (E-H) FoxM1 immunohistochemistry on Lep\(^{ob/ob}\) pancreas sections. (E) Arrowheads indicate some FoxM1\(^{LO}\) islet cells; arrow indicates FoxM1\(^{HI}\) islet cell (200X). Lines denote boundaries between islet and exocrine tissue. (F) Arrows indicate FoxM1\(^{HI}\) cells (100X). (G-H) FoxM1 and Ki67 double immunohistochemistry on Lep\(^{ob/ob}\) pancreas sections showing proliferating cells in an islet (G) and in acinar tissue (H) (200X). Arrows indicate double-positive cells. Lines denote boundaries between islets and exocrine tissue. (I) Model depicting FoxM1 regulation of optimal \(\beta\)-cell function and proliferation.
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**SUPPLEMENTARY FIGURE LEGENDS**

**Supplementary Figure 1.** Derivation of Foxm1<sup>LCA/LCA</sup> mice. (A) Exons 2-4 of the Foxm1 locus were replaced with a floxed selection cassette using BAC recombineering. Regions of homology used for BAC recombineering are designated as letters A-D. (B) Southern blots of ES cell colonies. DNA was digested with either XhoI or FspI and probed outside of the homology arms (see A). Colonies 2, 4, 5 and 6 were correctly targeted on the 3’ end (12 Kb) but only colonies 2, 5 and 6 were correctly targeted on the 5’ end (9 Kb).

**Supplementary Figure 2.** Islet size distribution at twelve months of age. Number of islets, binned by size and normalized to total pancreas area (n=5-6).

**Supplementary Figure 3.** Foxm1<sup>LCA/LCA</sup> mice display endoreplication in liver and heart. (A-F) DAPI staining on sections of E15.5 whole embryos. (A-B) Overview of heart (He), liver (Li), and lung (Lu) in WT (A) and Foxm1<sup>LCA/LCA</sup> (B) mice. (C-F) Higher magnification of liver (C-D) and heart (E-F). Cells with endoreplication are indicated with arrows and shown in higher magnification in insets in D and F.

**Supplementary Figure 4.** Liver (A) and heart (B) weight at E15.5 in Foxm1<sup>LCA/LCA</sup> and WT embryos. n=4-25; *p<0.05.
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