Cyclin D2 is essential for the compensatory β-cell hyperplastic response
to insulin resistance in rodents

Senta Georgia[1] Charlotte Hinault[2], Dan Kawamori[2], Jiang Hu[2], John Meyer[2], Murtaza Kanji[1],
Anil Bhushan[1#] and Rohit N. Kulkarni[2#]

[1] Larry Hillblom Islet Research Center, UCLA David Geffen School of Medicine, Los Angeles, CA 90095; [2] Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston MA 02215

* S.G. and C.H. contributed equally

Address correspondence to:
Rohit N. Kulkarni, Rohit.Kulkarni@joslin.harvard.edu;
or Anil Bhushan, abhushan@mednet.ucla.edu.

*Objectives***:** A major determinant of the progression from insulin resistance to the development of overt type 2 diabetes is a failure to mount an appropriate compensatory β-cell hyperplastic response to maintain normoglycemia. We undertook the present study to directly explore the significance of the cell cycle protein, cyclin D2, in the expansion of β-cell mass in two different models of insulin resistance.

Research Design and Methods: We created compound knockouts by crossing mice deficient in cyclin D2 (D2KO) with either the insulin receptor substrate-1 knockout (IRS1KO) mice or with the insulin receptor liver-specific knockout mice (LIRKO), neither of which develops overt diabetes on its own due to robust compensatory β-cell hyperplasia. We phenotyped the double knockouts and used RT-qPCR and immunohistochemistry to examine β-cell mass.

Results: Both compound knockouts, D2KO/LIRKO and D2KO/IRS1KO, exhibited insulin resistance and hyperinsulinemia and an absence of compensatory β-cell hyperplasia. However, the diabetic D2KO/LIRKO group rapidly succumbed early compared to a relatively normal lifespan in the glucose intolerant D2KO/IRS1KO mice.

Conclusions: This study provides direct genetic evidence that cyclin D2 is essential for the expansion of β-cell mass in response to a spectrum of insulin resistance and points to the cell cycle protein as a potential therapeutic target that can be harnessed for preventing and curing type 2 diabetes.

The maintenance of an adequate and functional pancreatic β-cell mass dictates the body's ability to compensate for insulin resistance. Recent studies in autopsy samples from humans reported expansion of β-cell mass from infants through adolescence that was largely due to increased islet size (1). Further, humans with established type 2 diabetes exhibit a deficit in β-cell mass in comparison to their non-diabetic cohorts (2;3) . Interestingly, obese, non-diabetic patients, express a wide range of β-cell mass that is sufficient to maintain euglycemia up to a specific threshold, and crossing the threshold correlates with impaired fasting glucose and clinical diabetes (4).

While direct data in humans is lacking, studies in rodents clearly indicate that β-cell mass adaptively expands to compensate for both physiological and pathophysiological states of insulin resistance including pregnancy, onset of obesity, high fat feeding and after partial pancreatectomy (5-9). Although the adaptation is dependent on alterations in both the number and size of β-cells and generation of new β-cells from endogenous progenitors (10;11), recent studies point to replication as a primary mechanism for the physiological maintenance of adult β-cell mass (12) and in response to insulin resistance in both rodents and humans (9;12-15).

Replication is achieved by re-entry of the β-cell into the cell cycle and relies on proteins regulating the G1 phase (13;16-19). Our previous work has established that cyclin D2, a G1/S cell cycle regulator, is necessary for the post-natal expansion of β-cell mass (13). In these studies we observed that in the absence of cyclin D2, the diminished β-cell mass established during the neonatal remodeling period was inadequate to sufficiently respond to metabolic demand for insulin in adult mice leading to glucose intolerance but did not lead to frank diabetes (13). While these experiments indicate that cyclin D2 is important during early postnatal expansion of β-cells for the adult mouse to achieve its optimal β-cell mass, its importance in cell expansion in response to pathophysiological demand for insulin is not known. Considering these observations, we explored whether a limited but adequate β-cell mass that is challenged by physiological stress would fall below a "functional threshold" required to maintain euglycemia, and eventually to promote the development of diabetes using cyclin D2 knockout mice. To this end, we created compound double knockouts by breeding cyclin D2 (D2KO) mice with either mice that are deficient in IRS1 (IRS1KO) (20;21), or mice with a knockout of the insulin receptor specifically in liver (LIRKO) (22). These models reflect the spectrum of insulin resistance and glucose intolerance observed in humans but do not develop frank diabetes, in part, due to compensatory β-cell expansion that can increase from 3-fold (IRS1KO) to 30-fold (LIRKO), largely by replication of β-cells (21;23).

Our results indicate that both D2KO/LIRKO and D2KO/IRS1KO double knockout mice fail to show a β-cell compensatory response to insulin resistance leading to overt diabetes that is secondary, in part to a dramatic decrease in β-cell mass due to reduced β-cell replication. These data provide genetic evidence that cyclin D2 is essential for the compensatory increase in β-cell hyperplasia in response to insulin resistant states.

MATERIALS AND METHODS
Animal breeding and genotyping: All studies and procedures were performed after approval by Institutional Animal Committee Regulations in both institutions. The creation and characterization of the

LIRKO, IRS1KO, and D2KO mice has been described previously (20-22;24). All mice were maintained on a 12-hour light/ 12-hour dark cycle with *ad libitum* access to water and food and a mixed mating scheme was adopted for breeding. In the first group, males and females homozygous for loxP sites (IRLox) and expressing Cre recombinase on an albumin promoter were bred with mice lacking cyclin D2. In the second group, males and females carrying either the IRS1 or cyclin D2 alleles were used. In the D2KO/LIRKO study, the breeding generated mice homozygous for loxP (controls), LIRKO, D2KO/LIRKO and D2KO and in the D2KO/IRS1KO study, the genotypes included IRS1KO (*cycD2+/-;irs1-/-*), D2KO/IRS1KO (*cycD2-/-;irs1-/-*), D2KO (*cycD2-/-;irs1+/-*), and their controls (*cycD2+/-;irs1+/-*). DNA extracted from tails was used for PCR-based genotyping (13;22). All studies included animals from a mixed genetic background. Pancreas were collected for immunohistochemical analyses (25) and islets were isolated using previously described methods (26).

Glucose and Insulin tolerance tests and plasma insulin levels: Following a 16-h fast, baseline blood glucose levels were measured from tail-vein samples using a glucometer (LifeScan, Milpitas, CA). Glucose tolerance tests were performed (25) in 7 week-old (D2KO/LIRKO) or 25 week-old mice (D2KO/IRS1KO). Plasma insulin levels were measured by ELISA (Linco Research). Insulin tolerance tests were performed on 25 week-old mice (25).

Real-Time RT-qPCR: Quantitative real-time RT-qPCR was performed on total RNA samples extracted from islets (RNeasy Mini Kit, Qiagen, Valencia, CA). cDNA samples were amplified by using SYBR Green PCR Master Mix (Applied Biosystems) and analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). All primer sequences are available in the supporting document which can be found in an online appendix at http://diabetes.diabetesjournals.org.

Immunohistochemistry and β-cell mass analyses: The pancreas were processed for immunohistochemical analyses as described earlier (25). Primary antibodies included: mouse anti-glucagon 1:1,000 (Sigma); guinea pig anti-insulin 1:500 (Dako); rabbit anti-somatostatin, 1:50 (Abcam); rabbit anti-Ki-67 1:50 (BD Biosciences), cyclin D1, 1:800 (Santa Cruz Biotechnology Inc.); rabbit anti–cyclin D2, 1:4,000 (Santa Cruz Biotechnology Inc.); cyclin D3, 1:200 (Santa Cruz Biotechnology Inc.); goat anti-PDX-1, 1:300 (gift from C.V Wright PhD, Vanderbilt U); rabbit anti-FoxO1, 1:50 (cell signaling); rabbit anti-Glut2 1:100 (Alpha Diagnostic). DAPI (Sigma-Aldrich) was used for nuclear staining. β-cell size was assessed by quantifying the area of cells that co-stained with β-catenin (mouse monoclonal antibody, BD Biosciences) and insulin using ImageJ software (NIH; http://rsb.info.nih.gov/ij/). β-cell mass was analysed as reported earlier (27).

Statistical analyses: All data are expressed as means ± SEM. Statistical significance was determined by unpaired Student's '*t*' test or analysis of variance (ANOVA) as appropriate and a P value <0.05 was used to reject the null hypothesis.

RESULTS

D2KO/LIRKO mice exhibit severe hyperglycemia and early diabetes. We crossed the LIRKO mice with D2KOs to generate compound double knockouts to investigate the requirement of cyclin D2 in the β-cell compensatory response to insulin resistance. Qualitatively similar data were obtained in females and males. Therefore, only data from males are reported. All mice were born in a normal Mendelian ratio and we did not observe embryonic lethality. However, we observed an early lethality in

the double knockouts with several mice dying by age ~3 months. Therefore, we phenotyped 7 week-old animals and used 10 to 12 week-old mice for islet isolation for gene expression or dissected the pancreas for immunohistochemistry.

No significant differences were observed in body weights between LIRKO and control mice (table 1- upper part) as reported previously (22). Consistent with previous data (23), LIRKOs exhibited hyperinsulinemia compared to controls (fig. 1A), and no significant differences were observed in blood glucose between groups in 10-12 week-old mice. The D2KOs were smaller than the controls and exhibited mild hyperglycemia that was secondary to hypoinsulinemia (table 1- upper part, fig. 1A). On the other hand, the compound double knockouts showed a significant decrease in body weight, severe hyperglycemia (>600 mg/dl) (table 1- upper part), and hyperinsulinemia, although their absolute insulin levels were still significantly lower compared to individual LIRKO mice (fig. 1A). Further, the circulating glucagon levels were significantly increased in the double knockouts compared to all other groups (fig. 1B).

Our previous studies have shown that D2KO animals were glucose intolerant, but were not severely diabetic despite a diminished β-cell mass (13). In this study, we observed severe intolerance as early as 7 weeks of age in both the LIRKO and D2KO/LIRKO mice compared either to the controls or to the mildly glucose intolerant D2KOs (fig. 1C). These data indicate that absence of cyclin D2 in LIRKO mice leads to overt diabetes and early death in the double mutants.

Absence of cyclin D2 limits islet hyperplasia in LIRKO mice. To evaluate the alterations in islet morphology, we analyzed pancreas sections by immunohistochemistry using a cocktail of antibodies to non β-cell hormones (fig. 2B). In parallel, we measured hormone gene expression using quantitative real time RT-qPCR, and these results are presented in fig. 2A. The significant increase in insulin gene expression observed in LIRKO islets was lost in the double knockout islets (fig. 2A) and was consistent with the relatively lower circulating insulin levels in the double knockouts (fig. 1A). On the contrary, the reduced glucagon gene expression observed in LIRKO islets was not detected in D2KO/LIRKO islets and in fact the circulating glucagon levels were increased in the double knockouts (fig. 1B). Further, the decrease in somatostatin gene expression in the LIRKO group was attenuated in the D2KO/LIRKO islets.

Examination of pancreas sections revealed a normal islet architecture and cell distribution in all groups (fig. 2B). As expected, we observed robust islet hyperplasia in the LIRKO mice compared to islet hypoplasia in the D2KO pancreas. Despite the hyperinsulinemia and insulin resistance, the D2KO/LIRKO mice failed to show islet hyperplasia and presented a morphology that was largely similar to that observed in D2KO islets.

While the size of islets is increased in the LIRKO group compared to controls, it was reduced in D2KO/LIRKO and D2KO mice (fig. 2 B-C). This absence of hyperplasia in D2KO/LIRKO islets was associated with a dramatic decrease in β-cell mass (fig. 2D). Consistent with our previous report (23), LIRKO islets showed a 2.4-fold increase in their β-cell mass compared to controls due to hyperplasia (fig. 2F) rather than hypertrophy (fig. 2E). Interestingly, quantification of β-cell size revealed a slight but significant increase in the double mutants suggesting an attempt to compensate for the reduced β-cell mass (fig. 2E). Therefore, to assess whether the absence of β-cell hyperplasia in D2KO/LIRKO pancreas was due to the inability of these cells to replicate in the

absence of cyclin D2, we analyzed the expression of Ki-67, a marker of replication, by immunohistochemistry and measured the replication index by computing the ratio of Ki-67$^+$/insulin$^+$ double positive cells to the total number of insulin+ cells (fig. 2F). A decrease in replicating β-cells in the double knockouts compared to LIRKO islets suggested that the loss of hyperplasia in the D2KO/LIRKO mice is, in part, secondary to reduced β-cell proliferation. These data indicate that absence of cyclin D2 leads to a significantly reduced β-cell replication and a poor islet hyperplastic response to insulin resistance. Evaluation of cell death by TUNEL immunostaining did not reveal significant differences between groups (data not shown).

Further evidence for a role for cyclin D2 in β-cell proliferation (13;28) emerges from our studies in the hyperplastic islets isolated from 4-6 month-old insulin resistant LIRKO mice (22). We observed a significant increase in transcripts for all three cyclins that was consistent with enhanced β-cell replication in the LIRKOs (fig. 3A). Further, immunostaining confirmed the expression of cyclins at the protein level with cyclin D2 showing a more intense nuclear staining compared to controls (fig. 3B).

Regulation of PDX-1 and GLUT2 in D2KO/LIRKO islets. We subsequently focused on examining the expression of FoxO1 (forkhead box 1) and PDX-1 (pancreas duodenum homeobox-1), two proteins known to be linked to β-cell proliferation (reviewed in (14)(29-32). Consistent with the previous report that PDX-1 is required for the compensatory β-cell growth response to insulin resistance (14), PDX-1 gene expression was significantly lower in the islets isolated from D2KO/LIRKO or D2KO mice compared to controls (fig. 4A). Further, PDX-1 protein was either virtually undetectable by immunostaining or severely reduced in

D2KO/LIRKO β-cells in most of the islets from these two groups (fig. 4B). On the other hand, islet gene expression for FoxO1 was not significantly different between groups (data not shown). However, evaluation of FoxO1 protein levels by immunostaining showed little nuclear but largely cytoplasmic staining in β-cells in the D2KO/LIRKO and D2KO islets compared to LIRKO or control islets (fig. 4B).

Considering that PDX-1 is known to regulate several β-cell-specific genes (33;34), we analyzed the expression of the glucose transporter GLUT2, a key marker of β-cell function by quantitative real-time RT-qPCR and immunostaining. GLUT2 gene expression was decreased in islets from each knockout group but more severely reduced in D2KO/LIRKO islets (fig. 4C), which correlated with the severe hyperglycemia in these mice compared to single knockout counterparts (table 1 - upper part). At the protein level, GLUT2, like PDX-1, was either virtually absent or severely reduced in D2KO/LIRKO β-cells (fig. 4D), thereby implicating a role for the transporter in the worsening of diabetes in the D2KO/LIRKO mice. These data indicate that a deficiency of cyclin D2 in the context of insulin resistance is associated with altered expression of PDX-1 and its downstream targets with potential implications for poor β-cell proliferation and function.

D2KO/IRS1KO mice manifest hypoinsulinemia, glucose intolerance, and hyperglycemia. In a parallel study, we crossed D2KO mice with another model of insulin resistance, the IRS1KOs, which exhibit mild insulin resistance and a compensatory increase in β-cell mass to maintain euglycemia (20;21). For clarity, we will refer to *cycD2$^{-/-}$;irs1$^{+/-}$* animals as D2KO, *cycD2$^{+/-}$;irs1$^{-/-}$* as IRS1KO, *cycD2$^{+/-}$;irs1$^{+/-}$* double heterozygotes as control, and *cycD2$^{-/-}$;irs1$^{-/-}$* as double knockouts (D2KO/IRS1KO). The IRS1KO animals exhibited reduced body

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mass at weaning and were easily identifiable (20). The D2KO/IRS1KO animals were generally smaller than IRS1KO or D2KO animals (table 1- lower part). By age 25 weeks, D2KO/IRS1KO animals remained smaller than their IRS1KO counterparts, while there was no significant difference in the body weights of the controls and D2KO animals (table 1-lower part). Thus the body mass phenotype of the conventional IRS1KO (20;21) was also maintained in the compound D2KO/IRS1KO mice.

Insulin resistance in the individual IRS1KO and D2KO/IRS1KO mice was confirmed by insulin tolerance tests in 25 week-old animals (fig. 5A). We then evaluated the ability of the D2KO/IRS1KO animals to secrete insulin in response to a glucose challenge. Serum insulin levels measured before and 30 minutes after intraperitoneal glucose injection revealed that unlike the hyperinsulinemic response in the IRS1KO animals, D2KO/IRS1KO animals failed to show substantive insulin secretory response to injected glucose, which was similar to the hypoinsulinemic response to glucose challenge observed in the D2KO group (fig. 5B). Taken together, these results indicate that D2KO/IRS1KO animals are as insulin resistant as their IRS1KO counterparts, but fail to show robust compensatory hyperinsulinemia.

Consistent with previous reports (20), glucose disposal measured by glucose tolerance tests in IRS1KO animals was largely similar to control animals (fig. 5C). In contrast, D2KO/IRS1KO animals were more severely glucose intolerant than the D2KO group, with blood glucose levels above 400mg/dL two hours after glucose challenge (fig. 5C). To confirm that these animals have overt diabetes (defined as non-fasting blood glucose levels >250mg/dL (27)), we measured the non-fasting blood glucose levels measured over a period of three days. At age 25 weeks, D2KO animals exhibited mild hyperglycemia, while IRS1KO and control animals showed euglycemia, and D2KO/IRS1KO animals were severely diabetic with blood glucose levels approaching 400mg/dL (table 1-lower part).

Diabetic D2KO/IRS1KO animals have disrupted islet architecture and severely diminished β-cell mass due to poor proliferation. We next examined islet architecture, β-cell mass and islet density in all groups. Consistent with previous reports, IRS1KO animals exhibited hyperplastic islets (20;35), while D2KO mice showed smaller islets compared to controls although the islet architecture was normal (13;28). The islets of D2KO/IRS1KO mice were smaller than D2KO islets, exhibited abnormal architecture, and had visibly degranulated β-cells (fig. 6A). Quantification confirmed a significant reduction in β-cell mass in the D2KO/IRS1KO animals (fig. 6B) that was more severe than their D2KO counterparts. There were no significant changes in α-cell mass (data not shown). Measurement of the diameter of individual β-cells revealed slightly larger β-cells in the D2KO/IRS1KO group, suggesting an attempt at compensation to counter the decreased β-cell mass (fig. 6C). Taken together, these data suggest that impaired β-cell replication likely accounted for the decrease in β-cell mass in the D2KO/IRS1KO mice.

To assess whether the diminished β-cell mass of D2KO/IRS1KO pancreas was due to an inability of β-cells to replicate due to the absence of cyclin D2, we measured the -cell proliferation index as for the D2KO/LIRKO study. Since β-cell replication declines with age, we chose to examine younger 6 to 8 week-old mice (36;37). IRS1KO β-cells showed a higher proliferation index in contrast to a significantly lower index in D2KO and D2KO/IRS1KO β-cells underscoring the importance of cyclin D2 for β-cell replication in this model (fig 6D). The absence of TUNEL+ β-cells in 6 to 8-week-

old mice in the D2KO/IRS1KO group suggested that apoptosis is unlikely to contribute to diminished β-cell mass at this age in the double mutants. However, in 25 week-old mice a higher rate of apoptosis was detectable in the D2KO and D2KO/IRS1KO islets consistent with a recent report (38) although these differences did not reach statistical significance (data not shown). Taken together, our data indicate that cyclin D2 is required for replication-dependent expansion of β-cell mass in response to insulin resistance.

Up regulation of expression of alternative D-type cyclins fails to initiate proliferation in replication-competent β-cells. We next explored whether the absence of cyclin D2 in D2KO/IRS1KO animals resulted in an upregulation of other members of the D-type cyclin family using immunohistochemistry (fig. 7). In control and IRS1KO pancreas sections, cyclin D1 was weakly expressed in both endocrine and exocrine cells (fig 7 (A,C)), and cyclin D3 (fig. 7 (I, K)) was absent. In the D2KO pancreas, cyclin D1 was detected in both endocrine and exocrine cells (fig. 7 (B)), and cyclin D3 was weakly expressed in the nuclei of some β-cells (fig.7 (J)). In contrast, in the D2KO/IRS1KO pancreas cyclin D1 expression was present (fig. 7 (D)), and cyclin D3 was expressed strongly in virtually all nuclei compared to a low intensity expression in D2KO pancreas (fig. 7 (L)). The enhanced expression of cyclin D3 and the presence of cyclin D1 in D2KO/IRS1KO animals indicated that despite their structural redundancy, these two D-type cyclins were unable to compensate for the absence of cyclin D2, further reinforcing the conclusion that cyclin D2 is essential for expansion of β-cell mass in response to insulin resistance.

DISCUSSION

The presence of an adequate functional β-cell mass is critical for maintaining euglycemia in mammals. In states of altered metabolic demand *e.g.* pregnancy or high fat feeding, healthy β-cells maintain euglycemia either by increasing insulin secretion or by an elevated β-cell mass or both (39). Indeed less than 20% of obese insulin-resistant individuals develop type 2 diabetes and a majority of insulin resistant humans are capable of maintaining euglycemia by β-cell compensation (9;40). Recent studies focused on genomic analyses of type 2 diabetic patients have reported polymorphisms in genes that are close to those coding for key cell cycle regulators (41;42); these experiments suggest that expansion of β-cell mass are linked to proteins in cell cycle progression. In this study we provide direct genetic evidence that the cell cycle protein, cyclin D2, is essential for compensatory β-cell expansion in response to insulin resistance.

We have reported earlier that cyclin D2 is essential for the physiological remodeling of β-cell mass in the post-natal period (13;28). To address if cyclin D2 is also required for β-cell expansion in states of insulin resistance, we crossed the cyclin D2 knockouts with two mouse models that exhibited varying degrees of insulin resistance. We chose the LIRKO model because it is tissue-specific and is characterized by severe resistance and a dramatic β-cell hyperplastic response (22). In the second model we used mice lacking IRS-1, which exhibited mild insulin resistance but still manifested islet hyperplasia (20;21). The presence of a significantly increased number of proliferating β-cells in the islets from both models indicates the presence of hyperplasia, in part, due to replication. While imposition of cyclin D2 insufficiency led to diabetes in both models, severe hyperglycemia was evident in the D2KO/LIRKO compound KOs at a much earlier age suggesting that the severity of insulin resistance, and the availability of cyclin D2, are important

determinants of an appropriate islet hyperplastic response. On the other hand, in the IRS1KO mice which develop mild to moderate post-receptor insulin resistance (20), the superimposition of cyclin D2 insufficiency also led to the development of diabetes. However the compound D2KO/IRS1KO mice continued to live until age 25 weeks and more closely mimicked the human progression of insulin-resistance mediated type 2 diabetes compared to the D2KO/LIRKO model. Further, the reduced expression of two key β-cell specific markers, PDX1 and GLUT2, suggest that absence of cyclinD2, in the context of insulin resistance, can directly influence β-cell function and proliferation.

Upstream signaling pathways − *e.g.* insulin/IGF-I (insulin like growth factor-I) − are crucial in the regulation of β-cell replication (see review (9)), and may be linked with cyclin D2 in modulating β-cell mass. Indeed, we previously reported that increased β-cell replication in LIRKO mice correlates with increased insulin levels but not with glucose levels (23). Furthermore, in this same study, we crossed LIRKO mice with β-cell specific insulin receptor knockout (βIRKO) mice, a model which exhibits β-cell hypoplasia and manifest a phenotype resembling human type 2 diabetes (25). Consistent with a role for the insulin receptor in modulating β-cell proliferation, βIRKO/LIRKO mice failed to develop islet hyperplasia and died as early as 8 weeks of age (23). Interestingly, islets and -cells derived from βIRKO mice show reduced cyclin D2 protein expression (unpublished data, Hinault and Kulkarni), and it is possible that this absence of the cell cycle protein contributes to poor islet growth and development of age-dependent diabetes in these mutants (43;44). Recent experiments have also linked the cyclin/CDK4 complex to Akt in β-cell proliferation indicating that Akt1

upregulates cyclin D1 and cyclin D2 levels, and CDK4 activity (45).

Cyclin/CDK complexes are key nodes in the regulation of the G1 to S transition during cell cycle progression. For example, global knockouts of CDK4 in mice develop β-cell hypoplasia and diabetes (16;17). Previous work by our own group has shown that mice with a global knockout of p27, a cell cycle inhibitor, regenerate β-cells more efficiently following STZ-induced diabetes (13), while other investigators have reported that β-cell specific over-expression of p27 leads to islet hypoplasia and diabetes (46). Conversely, the cell cycle inhibitor p21, which is the main target of the tumor suppressor p53, has been recently reported not to be essential for maintaining β-cell mass or function *in vivo* (47). In a similar manner, β-cell specific deletion of pRb, a protein reported to be a central regulator of the cell cycle, leads to minimal defects in β-cell replication, mass and function (48). Therefore, specific cell cycle regulators play a critical role in β-cell proliferation regardless of the redundant expression of complementary family members. Intriguingly, Lavine at al, reported an inability to detect cyclin D2 protein in proliferating human islets that over-expressed pre-pro-cholecystokinin (49). Whether human -cells utilize cyclin D2 for proliferation in a context-dependent manner or are dependent on another key cyclin protein requires further investigation.

While prior studies support a role for PDX-1 in β-cell proliferation, it is unclear whether proteins in the cyclin/CDK pathway are direct targets of this transcription factor. On the other hand, in our study, the decreased β-cell mass in the compound knockouts and reduced expression of PDX-1 suggests that cyclin D2 is upstream of the pancreas-duodenum homeodomain transcription factor. It is likely there are intermediates that link these two important proteins and additional

studies are warranted to define their roles in the regulation of β-cell proliferation.

It is worth noting that the cyclin D2 mutants were global knockouts and therefore it is possible that lack of cyclin D2 in putative precursors contributing to neogenesis also depend on cyclin D2-mediated cell cycle re-entry to facilitate enhanced β-cell mass. Nevertheless, our conclusion that cyclin D2 is necessary for replication-based -cell expansion in insulin resistant states is supported by lineage trace studies which provide firm evidence that self-duplication is the primary source of new β-cells in adult rodents (12). Taken together our work supports the concept that cyclin D2, or its analogue in humans, is a potential therapeutic target that can be harnessed for promoting β-cell expansion in the treatment of type 1 diabetes and to delay the progression and/or prevent the development of type 2 diabetes.

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Table 1

		Body Weight (g)	'p' Value (vs Control)	Non fasted blood glucose (mg/dl)	'p' Value (vs Control)
10-12 weeks of age	**Control**	24.8 ± 1.5		149 ± 9	
	LIRKO	24.2 ± 1.7	**NS**	198 ± 40	**NS**
	D2KO/LIRKO	18.5 ± 1.4	**<0.01**	>600	**<0.000001**
	D2KO	19.7 ± 1.4	**=0.024**	188 ± 22	**=0.042**
25 weeks of age	**Control**	23.1 ± 0.9		172 ± 5	
	IRS1KO	18.4 ± 1.1	**=0.012**	176 ± 9	**NS**
	D2KO/IRS1KO	13.5 ± 0.4	**<0.005**	390 ± 41	**<0.005**
	D2KO	21.2 ± 0.8	**NS**	253 ± 18	**<0.005**

Means ±SEM (n=5-9 male mice)
NS= non significant

Figure legends

Figure 1: D2KO/LIRKO mice exhibit severe hyperglycemia and early diabetes. A-B. Plasma insulin (A) and glucagon (B) levels were measured at random fed state in control (white), LIRKO (light grey), D2KO/LIRKO (black) and D2KO (dark grey) mice (n=4-10). *, p<0.05 as indicated by bars. **C.** Glucose tolerance tests were performed in 7 week-old mice and blood glucose was measured at 0, 15, 30, 60 and 120 minutes after intra-peritoneal (i.p.) injection of glucose (2 g/kg body weight). Groups are indicated by symbols as shown. *, p<0.05 for *versus* controls, n=4-8.

Figure 2: Absence of cyclin D2 limits islet hyperplasia in LIRKO mice. A. Real time RT-qPCR was performed on RNA extracted from islets of control (white), LIRKO (light grey), D2KO/LIRKO (black) and D2KO (dark grey) mice (n=4-9). Results are normalized to TBP and expressed relative to controls. *, p<0.05 versus groups as indicated. **B.** Triple immunostaining for insulin (blue), somatostatin (green) and glucagon (red) on pancreas sections from control, LIRKO, D2KO/LIRKO and D2KO mice. A representative islet for each group at magnification 40x is presented (n=4). **C.** Relative islet size was assessed from triple immunostaining presented in B. Quantification with Image J software of islet area is presented (mean ±SEM from n ≥ 10 islets counted per mouse, n=4-6 in each group). **D.** β-cell mass was assessed as described in Methods (n=4-6). **E**. Relative β-cell size was assessed by co-immunostaining for β-catenin, insulin and DAPI in pancreas sections from control, LIRKO, D2KO/LIRKO and D2KO mice (n=4-6). Quantification with Image J software of relative β-cell area is presented (mean ±SEM from n ≥ 100 cells counted per mouse, n=4-6 in each group). **F**. β-cell proliferation was assessed by co-immunostaining for Ki-67, insulin and DAPI in pancreas sections from control (white), LIRKO (light grey), D2KO/LIRKO (black) and D2KO (dark grey) mice. Respective replication index is presented. *, p<0.05 versus groups as indicated, n=4-6. In all cases at least 2 to 3 pancreas sections were used for each animal.

Figure 3: Increased expression of cyclin D2 in LIRKO islets. A. Real time RT-qPCR was performed on RNA extracted from islets of LIRKO (light grey) or control (white) mice (n=4). Results are normalized to TBP and expressed relative to controls. * p<0.05 for LIRKO *versus* controls. **B**. Co-immunostaining of cyclin D2 (green) with insulin (red) and DAPI (blue) in pancreas sections from control and LIRKO mice. One to two representative islets for each group at magnification 40x are presented, n=3. In all cases at least 2 to 3 pancreas sections were used for each animal.

Figure 4: Regulation of PDX-1 and Glut2 expression in D2KO/LIRKO islets. A-C. Real time RT-qPCR was performed on RNA extracted from control (white), LIRKO (light grey), D2KO/LIRKO (black) and D2KO (dark grey) islets. Results are normalized to TBP and expressed relative to controls. * p<0.05 in comparison to controls as indicated, n=4. **B**. Co-immunostaining of PDX-1 (red) and FoxO1 (green) with DAPI (blue) in pancreas sections from control, LIRKO, D2KO/LIRKO and D2KO mice. A representative islet for each group at magnification 40x is presented (n=4). **D**. Co-immunostaining of GLUT2 (green) and insulin (red) with DAPI (blue) in pancreas sections from control, LIRKO, D2KO/LIRKO and D2KO mice. A representative islet for each group at magnification

40x is presented (n=4). In all cases at least 2 to 3 pancreas sections were used for each animal.

Figure 5: D2KO/IRS1KO animals are insulin resistant, hypoinsulinemic, and glucose intolerant. A. Insulin resistance was quantified by insulin tolerance tests. 0.75mU/g BW of insulin was injected i.p. and blood was collected from tail veins of control (white), D2KO (grey), IRS1KO (striped), and D2KO/IRS1KO (hatched) mice. Data are expressed as area under the curve of glucose excursion. **B.** Plasma insulin levels were measured before (white) and 30 minutes after (black) i.p. injection of glucose in control, D2KO, IRS1KO, or D2KO/IRS1KO mice. **C.** Glucose tolerance test. Blood glucose was measured at 0, 15, 30, 60 and 120 minutes after i.p. injection of glucose. $*p<0.05$, $**p<0.01$, $***p<0.005$ in comparison to controls (A-C) or as indicated by bars. Groups are indicated by symbols as shown, n=7-8.

Figure 6: D2KO/IRS1KO mice fail to exhibit compensatory β-cell replication and islet hyperplasia in response to insulin resistance. A. Co-immunostaining for insulin (green) and glucagon (red) in pancreas sections from control, D2KO, IRS1KO, and D2KO/IRS1KO mice as described in Methods. A representative islet for each group at magnification 20x is presented, n=3. **B.** Quantification of β-cell mass in control (white), D2KO (grey), IRS1KO (striped), and D2KO/IRS1KO (hatched) mice. *, p=0.03 compared to controls (n=3-5). **C.** Quantification of relative β-cell diameter. A minimum of 100 cells were measured per genotype. $*p<0.05$, $**p<0.01$, $***p<0.005$ in comparison to control. **D.** Quantification of the proliferation index of replicating β-cells for each group in control (white), D2KO (grey), IRS1KO (striped), and D2KO/IRS1KO (hatched) mice. *, p<0.05 compared to controls, n=3-5.

Figure 7: Alterations in expression of D-type cyclins in D2KO/IRS-1KO islets. Co-immunostaining for insulin (green) and cyclin (red) D1 or D2 or D3 in pancreas sections from control, D2KO, IRS1KO, and D2KO mice. A representative islet for each group at magnification 20x is presented, n=3.