ICA512 signaling enhances pancreatic -cell proliferation by regulating cyclins D through STATs

Mziaut, H.; Kersting, S.; Knoch, K.-P.; Fan, W.-H.; Trajkovski, Mirko; Erdmann, K.; Bergert, H.; Ehehalt, F.; Saeger, H.-D.; Solimena, M.

How to cite

MZIAUT, H. et al. ICA512 signaling enhances pancreatic -cell proliferation by regulating cyclins D through STATs. In: Proceedings of the National Academy of Sciences of the United States of America, 2008, vol. 105, n° 2, p. 674–679. doi: 10.1073/pnas.0710931105

This publication URL: https://archive-ouverte.unige.ch/unige:156960

Publication DOI: 10.1073/pnas.0710931105

ICA512 signaling enhances pancreatic β-cell proliferation by regulating cyclins D through STATs

Hassan Mziaut*, Stephan Kersting*†, Klaus-Peter Knoch*, Wan-Hung Fan*, Mirko Trajkovski*, Katja Erdmann*, Hendrik Bergert*†, Florian Ehehalt*†, Hans-Detlev Saeger†, and Michele Solimena*‡§¶

Departments of *Experimental Diabetology, †Surgery, and ‡Internal Medicine III, Center for Regenerative Therapies Dresden, School of Medicine, Dresden University of Technology, 01307 Dresden, Germany; and §Max Planck Institute for Molecular Cell Biology and Genetics, 01307 Dresden, Germany

Communicated by Pietro V. De Camilli, Yale University School of Medicine, New Haven, CT, November 20, 2007 (received for review June 12, 2007)

Changes in metabolic demands dynamically regulate the total mass of adult pancreatic β-cells to adjust insulin secretion and preserve glucose homeostasis. Glucose itself is a major regulator of β-cell proliferation by inducing insulin secretion and activating β-cell insulin receptors. Here, we show that islet cell autoantigen 512 (ICA512)/IA-2, an intrinsic tyrosine phosphatase-like protein of the secretory granules, activates a complementary pathway for β-cell proliferation. On granule exocytosis, the ICA512 cytoplasmic domain is cleaved and the resulting cytosolic fragment (ICA512-CCF) moves into the nucleus where it enhances the levels of phosphorylated STAT5 and STAT3, thereby inducing insulin gene transcription and granule biogenesis. We now show that knockdown of ICA512 decreases cyclin D1 levels and proliferation of insulinoma INS-1 cells, whereas β-cell regeneration is reduced in partially pancreatectomized *ICA512*$^{-/-}$ mice. Conversely, overexpression of ICA512-CCF increases both cyclin D1 and D2 levels and INS-1 cell proliferation. Up-regulation of cyclin D1 and D2 by ICA512-CCF is affected by knockdown of STAT3 and STAT5, respectively, whereas it does not require insulin signaling. These results identify ICA512 as a regulator of cyclins D and β-cell proliferation through STATs and may have implication for diabetes therapy.

diabetes | insulin | phosphatase | regeneration | secretion

The mass of insulin-producing pancreatic β-cells dynamically changes according to metabolic conditions. A close correlation exists between body weight, insulin demand, and β-cell number (1–3). This balance is achieved by β-cell formation through neogenesis or proliferation, and death by apoptosis or necrosis. In mice, expansion and differentiation of islet precursor cells accounts for all β-cell neogenesis until the first week of life (4, 5). Thereafter, replication of preexisting β-cells is the main, if not the only source, of new β-cells (6–8). Because increasing β-cell mass is important for treating diabetes, determining how β-cell proliferation is regulated is essential.

Growth hormone (GH), prolactin (PRL), and placenta lactogen (PL) foster β-cell proliferation (9–12). Binding to their receptors leads to JAK-mediated tyrosine phosphorylation and activation of STAT5a and 5b (13–15), which, in turn, up-regulate cyclin D2 expression (12). These findings are consistent with the role of STATs as potent mediators of mitogenic signals (16, 17). *STAT5a-* and *5b*-deficient mice, in particular, display defective cell proliferation associated with lack of expression of cyclins and cyclin-dependent kinases (18).

Recently, we identified a signaling pathway that couples exocytosis of insulin secretory granules with their biogenesis through STATs (19). This pathway involves the islet cell autoantigen 512/IA-2 of type 1 diabetes, which is enriched in the granule membrane (20). When granules fuse with the plasma membrane, the intracellular portion of the ICA512 transmembrane form (ICA512-TMF) is cleaved by calpain-1 (19, 21). The resulting cleaved cytosolic fragment (ICA512-CCF), which contains a catalytically inactive tyrosine phosphatase domain, translocates into the nucleus (19). There, it acts as a decoy phosphatase that binds to tyrosine-phosphorylated STAT5b and STAT3 to prevent their dephosphorylation by tyrosine phosphatases (22). By prolonging STATs activity, ICA512-CCF increases the expression of *insulin* and other granule genes (22). This positive effect of ICA512 on transcription is counteracted by the E3 SUMO-ligase PIASy, which sumoylates ICA512-CCF, thereby inhibiting the interaction of the latter with STAT5 (22). Here, we have extended these studies to investigate whether ICA512, by enhancing STAT activity, also affects β-cell proliferation and regeneration.

Results

ICA512 Enhances INS-1 Cell Proliferation. To test whether ICA512 affects the proliferation of INS-1 cells, we down-regulated its expression by RNA interference (RNAi). Two distinct silencing hairpins for *ICA512* were introduced into INS-1 cells by using the *pGENE-Clip* vector. Although each hairpin alone was only moderately effective at silencing ICA512 (supporting information (SI) Fig. 6), their combination decreased the levels of *ICA512* mRNA (Fig. 1*A*) and its products pro-ICA512 and ICA512-TMF (Fig. 1 *B* and *C*) by 56 ± 13.5%, 60 ± 13%, and 40 ± 5%, respectively. Knockdown of *ICA512*, in turn, decreased the percentage of proliferating, BrdU$^+$ INS-1 cells from 28 ± 7.8% to 14 ± 7.5% (Fig. 1 *D* and *E*). This reduction is especially significant considering the neoplastic nature of INS-1 cells and the partial knockdown of *ICA512* achieved with our transfection protocol, whose efficiency is ≈50–60% (19). The opposite effect was seen on overexpression of *ICA512-CCF* tagged with green fluorescent protein (*ICA512-CCF-GFP*) in INS-1 cells stimulated with 20 nM growth hormone to activate STAT5 and STAT3. After 8 h of labeling, *ICA512-CCF-GFP*$^+$ cells were increased by 65 ± 26% relative to BrdU$^+$ *GFP*$^+$ cells (Fig. 1*F*). Similarly, after labeling for 2 h, [^{3}H]thymidine incorporation was increased by 60 ± 23% in *ICA512-CCF-GFP*$^+$ INS-1 cells relative to *GFP*$^+$ cells (Fig. 1*G*). [^{3}H]Thymidine incorporation in *ICA512-CCF-GFP*$^+$ cells was still 21 ± 10% and 33 ± 13% higher than in *GFP*$^+$ cells after labeling for 10 and 24 h, respectively.

Deletion of *ICA512* Impairs β-Cell Regeneration. Because INS-1 cells have a higher mitotic index than β-cells, their use to investigate pathways regulating β-cell proliferation, while informative (23–25), should be accompanied by *in vivo* studies on pancreatic islets. To this aim, *ICA512*$^{-/-}$ mice and control littermates underwent partial pancreatectomy or total splenectomy (sham operation) (Fig. 2*A*). Removal of 70–80% of the pancreas is an established procedure

Author contributions: H.M. and S.K. contributed equally to this work; H.M., S.K., H.-D.S., and M.S. designed research; H.M., S.K., K.-P.K., W.-H.F., M.T., K.E., H.B., and F.E. performed research; H.M., S.K., K.-P.K., W.-H.F., M.T., and M.S. analyzed data; and H.M., S.K., and M.S. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

¶To whom correspondence should be addressed. E-mail: michele.solimena@mailbox.tu-dresden.de.

This article contains supporting information online at www.pnas.org/cgi/content/full/0710931105/DC1.

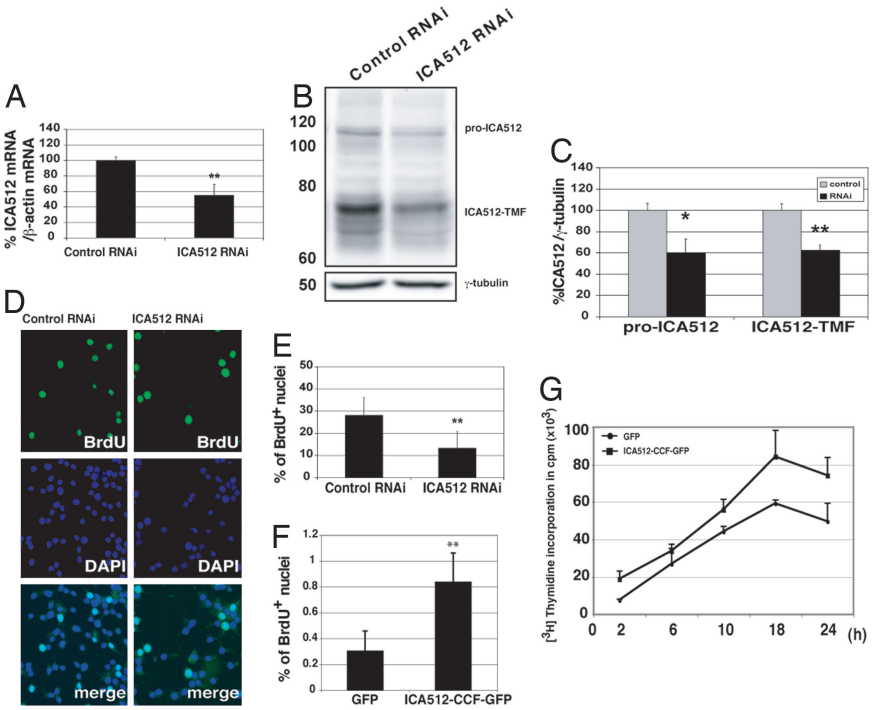

Fig. 1. ICA512 enhances proliferation of INS-1 cells. (*A*) *ICA512* mRNA levels as measured by real-time PCR in INS-1 cells transfected with two silencing hairpins for *ICA512* (*ICA512 RNAi*) in the *pGENEClip* vector or with the empty vector alone (*control RNAi*). Values were normalized for β-actin mRNA. (*B*) Western blot for pro-ICA512 *(Top)*, ICA512-TMF (*Middle*), and γ-tubulin (*Bottom*) on extracts from INS-1 cells transfected with control or ICA512 hairpins for RNAi. (*C*) Quantification of pro-ICA512 and ICA512-TMF, as shown in *B*. Values were normalized for γ-tubulin. (*D*) Fluorescence microscopy of INS-1 cells transfected with either control or ICA512 hairpins for RNAi. Four days after transfection cells were incubated with 5 μg/ml BrdU for 1 h and then immunostained with anti-BrdU (pseudogreen) and counterstained with DAPI (pseudoblue). (*E*) Percentage of BrdU$^+$ INS-1 cells transfected as in *D*. (*F*) Percentage of BrdU$^+$ INS-1 cells transfected either with *GFP* or with *ICA512-CCF-GFP*. The cells were synchronized by serum deprivation, and then stimulated with growth hormone for 20 min and harvested after 8 h. (*G*) Incorporation of [^{3}H]thymidine in INS-1 cells transfected and treated as in *F*. After stimulation with growth hormone, the cells were harvested at the indicated times. The data are representative of at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$.

that induces compensatory β-cell replication in the remaining pancreas to match insulin production and secretion with metabolic demand (7, 26–29). During the operation, we implanted an osmotic minipump in the abdomen that continuously released ≈25 μg of BrdU per hour for 7 days, thus ensuring that virtually every dividing cell was labeled during this time. One day after surgery, glycemia was slightly elevated in pancreatectomized *ICA512*$^{-/-}$ mice relative to pancreatectomized control mice (Fig. 2*B*). This finding is consistent with the notion that insulin secretion is impaired in *ICA512*$^{-/-}$ mice (30). This deficit, however, was transient, and by the end of the 1-week-long protocol, glucose levels were comparable between pancreatectomized *ICA512*$^{-/-}$ and control mice. There was also no difference in the glycemia of control and ICA512$^{-/-}$ sham-operated mice (SI Fig. 7). Seven days after surgery, mice were killed, the remnant pancreas excised, and β-cell renewal measured by counting BrdU$^+$ cells (Fig. 2*C*). The average number of β-cells per islet, as assessed by immunostaining for insulin, did not differ significantly among mice within the same group and among different groups (Table 1 and SI Table 2). BrdU$^+$ β-cells were comparably rare in sham-operated *ICA512*$^{-/-}$ and control mice (Fig. 2 *C* and *D* and Table 1). However, although the percentage of all BrdU$^+$ cells in pancreatectomized *ICA512*$^{-/-}$ (10.8%) and wild-type (11.2%) mice was comparable (SI Fig. 7 *B* and *C*), only 11 ± 1.2% of the insulin$^+$ cells were BrdU$^+$ in

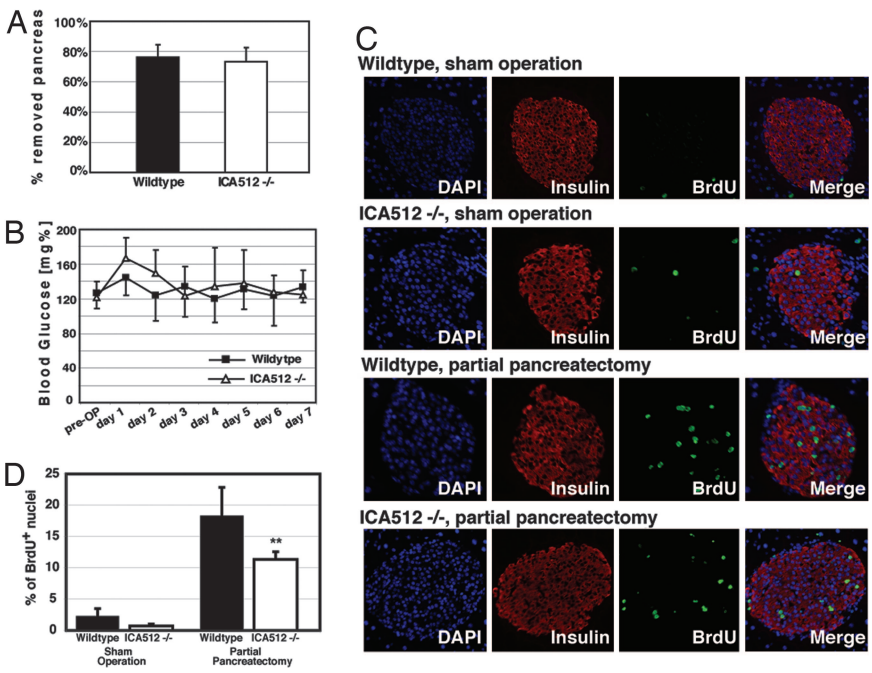

Fig. 2. β-cell regeneration is impaired in pancreatectomized ICA512$^{-/-}$ mice. (*A*) Extent of partial pancreatectomy in *ICA512*$^{-/-}$ mice and control littermates as measured by weighing the removed pancreatic tail at the time of surgery and the remnant pancreas at the time of euthanizing. (*B*) Blood glucose levels in *ICA512*$^{-/-}$ mice and wild-type mice before surgery (pre-OP) and on each of the following days as measured by blood sampling from the tail vein. (*C*) Immunomicroscopy on paraffin sections of the remnant pancreas from sham-operated and partially pancreatectomized *ICA512*$^{-/-}$ mice and wild-type mice after one week of continuous administration of BrdU. Sections were immunolabeled with anti-insulin (pseudored) and anti-BrdU (pseudogreen) antibodies. Nuclei were counterstained with DAPI (pseudoblue). (*D*) Percentage of insulin$^+$ and BrdU$^+$ cells. Three independent series with at least four mice per condition were used and at least five sections per mouse for a total of >50 islets were counted. **, $P < 0.01$.

Table 1. Complete data set for β-cell regeneration in sham-operated and partial pancreatectomized ICA512$^{-/-}$ and wild-type (WT) mice

Condition	Total no. of mice from three independent series	No. of counted islets per mouse	Total no. of counted islets	No. of β-cells counted	Mean no. of β-cells per islets	Mean per mouse	SD	No. of BrdU$^+$ β-cells	Mean per mouse	SD	BrdU$^+$ β-cells, %	SD
WT sham	4	50	200	11,430	57.15	2,858	322.30	2,858	66	36.01	2.31	1.2
ICA512−/− sham	4	50	200	12,259	61.30	3,065	298.28	3,065	22	7.80	0.73	0.3
WT pp	9	50	450	32,635	72.52	3,626	300.75	3,626	670	208.80	18.48	4.
ICA512−/− pp	9	50	450	29,854	66.34	3,317	346.05	3,317	378	72.01	11.40	1.2

pancreatectomized *ICA512*$^{-/-}$ mice compared with 18 ± 4.5% in pancreatectomized wild-type littermates (Fig. 2*C*) . Taken together, these data indicate that ICA512 promotes β-cell replication.

ICA512 Up-regulates Cyclin D1 and D2 Expression. STAT5a/b and STAT3 are the most relevant STATs for enhancing β-cell gene expression and proliferation (9, 10, 31). Activation of STAT3 and STAT5a/b by growth hormones, in particular, augments the expression of cyclin D1 (11) and D2 (12), respectively. These cyclins, in turn, induce the transition from G_1 to S phase in the cell cycle. Thus, we tested whether ICA512 enhances β-cell replication by increasing the expression of cyclin D1 and D2 through STAT5 and STAT3. In *ICA512-CCF-GFP*$^+$ INS-1 cells the levels of *cyclin D1* mRNA increased 2.5-fold relative to *GFP*$^+$ cells (Fig. 3*A*). In *ICA512-CCF-GFP*$^+$ INS-1 cells stimulated with 20 nM growth hormone, the protein levels of cyclin D1 and cyclin D2 and the amount of tyrosine-phosphorylated STAT5 (PY-STAT5) were also increased (Fig. 3*B*). An increment of cyclin D1 levels was also detected on expression of ICA512-CCF fused to a triple hemagglutinin (HA) epitope tag (*HA3-ICA512-CCF*) (SI Fig. 8), consistent with findings indicating that tagging of ICA512-CCF is not responsible for its nuclear translocation and regulation of gene transcription (19).

Next, we investigated the impact of ICA512 knockdown on STAT5 and cyclins D1 and D2 expression in INS-1 cells. Down-regulation of ICA512 by 40 ± 12% reduced the levels of nuclear STAT5b by 46 ± 5% relative to cells transfected with the control vector for RNA interference (Fig. 3 *C* and *D*). In ICA512 RNAi cells cyclin D1 was down-regulated by 39 ± 8%, but this decrease was seen only in resting conditions. Conversely, the levels of cyclin D2 were not significantly altered.

Both cyclin D1 and D2 are implicated in the regulation of β-cell mass (32), but evidence suggests that STAT5 enhances only the expression of cyclin D2 (9, 33). Thus, we asked whether up-regulation of cyclin D1 and D2 levels by ICA512-CCF is STAT5-dependent. To test this possibility, we down-regulated STAT5 expression in INS-1 cells either alone or in combination with ICA512-CCF-GFP overexpression. Knockdown of STAT5 by 70 ± 3% correlated with a 30 ± 8% and 36 ± 8% reduction in cyclin D1 and D2 levels, respectively (Fig. 4 *A* and *B* and SI Fig. 9), although it did not significantly affect STAT3 expression (Fig. 4 *A* and *B*). The reduction of cyclin D1 could result from the down-regulation of ICA512 levels secondary to the knockdown of STAT5 (23). On ICA512-CCF-GFP overexpression, instead, an equivalent down-regulation of STAT5 still correlated with the up-regulation of cyclin D1 by 200 ± 14%, whereas cyclin D2 levels were reduced by 56 ± 17% (Fig. 4 *A* and *B* and SI Fig. 9). These data suggest that ICA512-CCF up-regulates cyclin D2, but not cyclin D1, through STAT5.

ICA512-CCF Requires STAT3, but not Insulin, to Induce Cyclin D1 Expression. In addition to granule biogenesis and β-cell proliferation, ICA512-CCF enhances glucose-stimulated insulin secretion by disrupting the association of granules with the cortical actin cytoskeleton (M.T., H.M., S. Schubert, Y. Kalaidzidis, A. Krüger, and M.S., unpublished work). To test, therefore, whether its enhancement of cyclin D1 expression could be ascribed to the activation of insulin receptors on insulin release, we analyzed the impact of

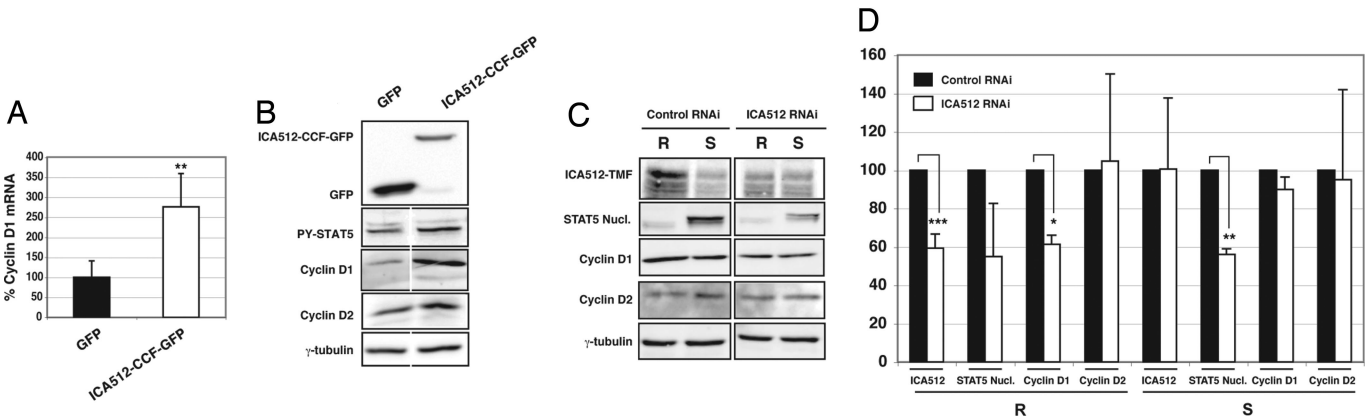

Fig. 3. ICA512-CCF up-regulates cyclin D1 expression. (*A*) The levels of *cyclin D1* mRNAs in *GFP*- or *ICA512-CCF-GFP* INS-1 cells were quantified by real-time PCR, normalized for β-*actin* mRNA. The amount of *cyclin D1* mRNA in *GFP*$^+$ INS-1 cells was equaled to 100%. Results shown are from three independent experiments performed in triplicate. (*B*) Western blots with anti-GFP, -PY-STAT5b, -cyclin D1, -cyclin D2, and -γ-tubulin antibodies on 20 μg of protein from *GFP* or *ICA512-CCF-GFP* INS-1 cells. Bands were cropped from a single gel. (*C*) Immunoblots for the indicated proteins from INS-1 cells transfected with *control* or both *ICA512* hairpins for RNAi. Three days posttransfection, INS-1 cells were cultured in serum-free media for 18 h, then either kept at rest, or stimulated with 25 mM glucose, 55 mM KCl, and 20 mM human growth hormone for 2 h. The cells were then incubated for additional 4 h in RPMI-1640 with 0.5% FBS before being harvested. (*D*) Quantification of ICA512, STAT5, cyclin D1, and cyclin D2 in INS-1 cells transfected and treated as in *C*. The protein levels were normalized to γ-tubulin and the amount of each protein from resting or stimulated cells transfected with the control hairpin for RNAi was equal to 100%. Results shown are from three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

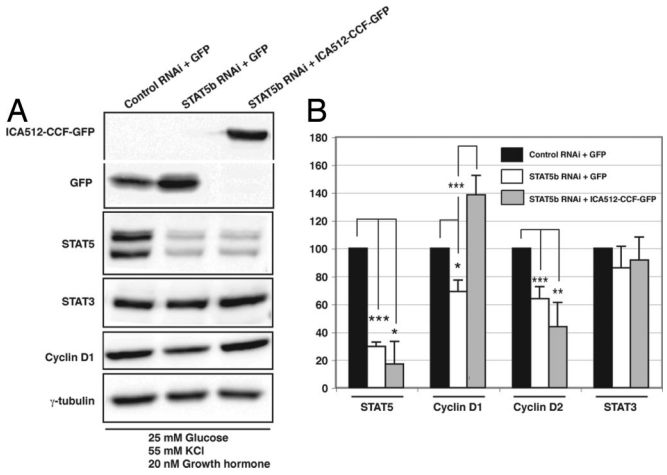

Fig. 4. ICA512 up-regulation of cyclin D1 expression is STAT5-independent. (*A*) Western blots with anti-GFP, -STAT5, -STAT3, -cyclin D1, and -γ-tubulin antibodies on 20 μg of protein from *GFP*$^+$ or *ICA512-CCF-GFP*$^+$ INS-1 cells cotransfected with *control* or *STAT5* hairpin for RNAi. Three days posttransfection, the cells were cultured in serum-free media for 18 h and then stimulated as described in Fig. 3*C*. (*B*) Quantification of STAT5, cyclin D1, and STAT3 in INS-1 cells transfected and treated as in *A*. Levels in cells cotransfected with the *control* hairpin for RNAi and *GFP* were equal to 100%. Results are from three independent experiments performed in triplicate. *, $P < 0.05$; ***, $P < 0.005$.

ICA512-CCF-GFP on cyclin D1 expression in STAT5-depleted INS-1 cells stimulated for 2 h, after 4 h in serum-free medium, with either 25 mM glucose, 25 mM glucose plus 55 mM KCl, or 20 nM growth hormone. Notably, the levels of cyclin D1 were greatly reduced when STAT5-depleted cells expressing ICA512-CCF-GFP were stimulated either with 25 mM glucose (Fig. 5*A,* lanes 4–6) or 25 mM glucose plus 55 mM KCl (Fig. 5*A,* lanes 1–3), two conditions that induce insulin secretion. Conversely, stimulation with 20 nM growth hormone alone (Fig. 5*A,* lanes 7–9) or together with 5 mM KCl (SI Fig. 10), that is, in conditions that elicit STAT signaling but not insulin release, increased the levels of cyclin D1, but not of cyclin D2. To directly test an involvement of insulin in the ICA512-CCF-mediated induction of cyclin D1, STAT5-depleted, GFP$^+$, or ICA512-CCF-GFP$^+$ INS-1 cells were again stimulated with 25 mM glucose, 55 mM KCl, and 20 nM growth hormone, this time in the constant presence or absence, even during the 4-h poststimulatory period, of an anti-insulin antibody for neutralization of secreted insulin. Intriguingly, the incubation with the anti-insulin antibody correlated with a decreased expression of cyclin D1, but did not preclude ICA512-CCF-GFP from enhancing the levels of cyclin D1 (Fig. 5*B*, compare lanes 4 and 6). Taken together these results suggest that signaling by growth hormone, but not insulin, is required for ICA512-CCF-mediated induction of cyclin D1 expression.

Based on these findings, we hypothesized that STAT3 is responsible for up-regulating cyclin D1 expression downstream of ICA512-CCF. We tested this hypothesis by reducing the levels of STAT3 by RNAi. However, depletion of STAT3 was accompanied by a compensatory up-regulation of STAT5, which can account for the concomitant increase of cyclin D1, and especially cyclin D2 (Fig. 5*C,* lane 2), relative to control cells D2 (Fig. 5*C*, lanes 2). Nevertheless, in these conditions the over-expression of ICA512-CCF was not associated with an increase of cyclin D1 levels (Fig. 5*C,* lane 3), suggesting that STAT3 is indeed critical for the ability of ICA512-CCF to up-regulate the expression of cyclin D1.

Discussion

It has recently become clear that β-cells proliferate under various physiological conditions and their turnover is controlled by numerous extrinsic and intrinsic factors (3). A partial list of positive regulators of β-cell proliferation includes incretins (34), lactogens and growth hormone (9), EGF + gastrin 1 (35), HNF-4a (36), calcineurin/NFAT (37), Wnt3a (38), and integrins (39). Not surprisingly, however, glucose is emerging as perhaps the major factor promoting the expansion of β-cell mass during adult life (40, 41). This effect has been attributed to the activation of β-cell insulin receptor/insulin receptor substrate-2 (IRS2) signaling by secreted insulin (42). This pathway, in particular, enhances the activity of the transcription factor Pdx-1 through the Akt-dependent inhibition of its repressor FoxO. Glucose metabolism may also influence this pathway by enhancing Ca^{2+}/calmodulin-dependent phosphorylation of CREB, which then up-regulates the expression of IRS2 (41, 43). Here, we provide the first evidence of a retrograde pathway for β-cell proliferation that is coupled to granule exocytosis, but is distinct from autocrine activation of insulin signaling.

Specifically, we show that knockdown of ICA512 expression reduces proliferation of INS-1 cells. Likewise, we demonstrate that regeneration of β-cells in pancreatectomized *ICA512* $^{-/-}$ mice is diminished. The effect of ICA512 on β-cell proliferation depends on the signaling function of ICA512-CCF, which is generated after granule exocytosis. Indeed, overexpression of ICA512-CCF is sufficient to enhance the proliferation of INS-1 cells. We have shown that ICA512-CCF increases the transcription of insulin and other granule components by preventing the dephosphorylation of PY-STAT5 and PY-STAT3, thus prolonging their transcriptional activity (22). Conceivably, STAT5 and STAT3 account also for the ability of ICA512-CCF to promote β-cell replication. STATs are latent transcription factors that are activated through tyrosine phosphorylation by cytokines, growth hormone, prolactin, and placental lactogen. Activated STATs translocate to the nucleus, where they enhance the expression of various genes. Previous studies have shown that activation of STAT5 by growth hormone promotes the proliferation of INS-1 cells through the induction of cyclin D2, but not cyclin D1 (12). STAT3, however, is a known inducer of cyclin D1 (11) and β-cell proliferation (44).

In mammalian cells the three types of cyclin Ds, namely D1, D2, and D3, play an essential role in promoting cell cycle progression from G_1 to S phase (45). Cyclin D1 and D2 are expressed in β-cells and regulate β-cell proliferation, whereas cyclin D3 is expressed at very low levels (32). Cyclin D2 is dispensable for expansion of total β-cell mass during mouse embryogenesis, but becomes essential for replication of neonatal β-cells (46), which, unlike pancreatic acinar and ductal cells, do not efficiently up-regulate other cyclins D (50). Cyclin D1 is also not required for β-cell development because, in *cyclin D1*$^{-/-}$ mice, islets are normal in number, size, and morphology (32). Overexpression of cyclin D1 in cultured islets, however, increases β-cell proliferation (47). Despite these findings, the signaling pathway that regulates cyclin D1 expression in β-cells is unknown.

Here, we show that overexpression of ICA512-CCF concomitantly with stimulation of INS-1 cells with growth hormone increases the levels of both cyclin D1 and cyclin D2. Down-regulation of STAT5 prevented ICA512-CCF from up-regulating the expression of cyclin D2, but not of cyclin D1. However, depletion of STAT3, despite the compensatory up-regulation of STAT5, precluded ICA512-CCF from enhancing cyclin D1 expression. Based on these findings, we suggest, therefore, that STAT3 and STAT5 mediate the positive regulatory role of ICA512-CCF on cyclin D1 and D2, respectively.

In summary, this study demonstrates that the C-terminal fragment of ICA512 generated on granules exocytosis, in addition to inducing insulin granule secretion (M.T., H.M., S. Schubert, Y. Kalaidzidis, A. Krüger, and M.S., unpublished work) and biogenesis (19, 22), promotes β-cell proliferation by converging with signaling by STAT5 and STAT3. This feedback pathway may allow β-cells to adjust their insulin output to the metabolic needs, especially in conditions of increased demand such as in pregnancy and obesity.

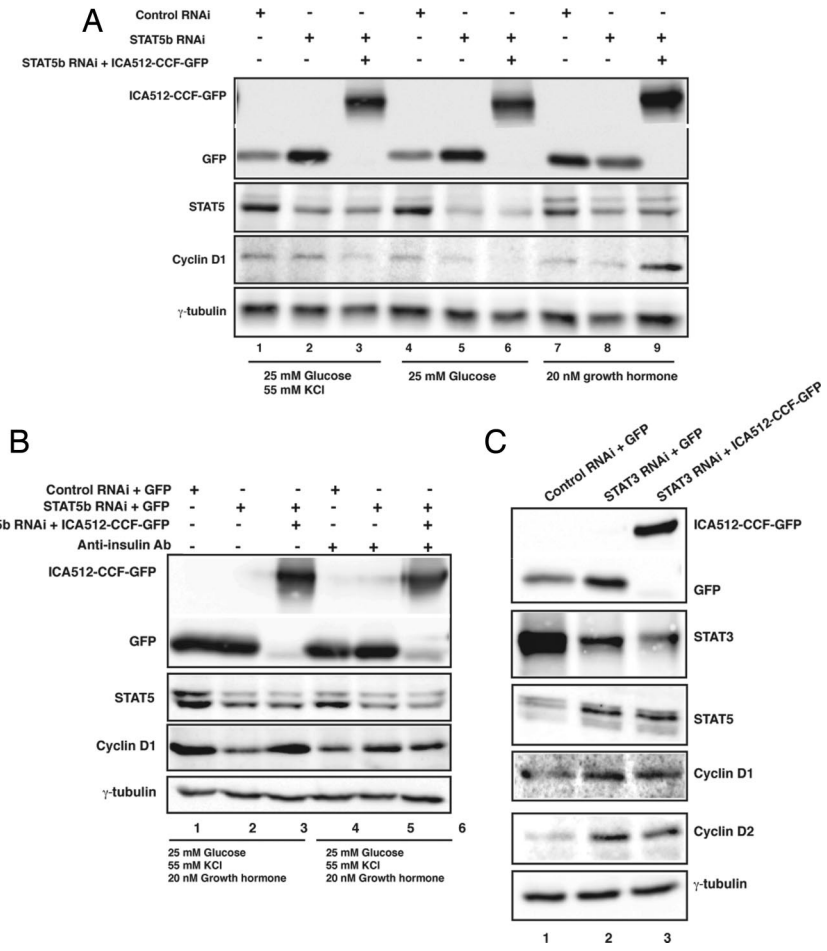

Fig. 5. Up-regulation of cyclin D1 by ICA512-CCF requires STAT3, but not insulin signaling. (*A*) Western blot with the indicated antibodies on INS-1 cells transfected as described in Fig. 4*A*. The cells were stimulated either with 25 mM glucose and 55 mM KCl, 25 mM glucose, or 20 nM growth hormone. (*B*) Western blot on INS-1 cells transfected as described in Fig. 4*A* and incubated either in the absence or presence of an anti-insulin neutralizing antibody. (*C*) Western blots with anti-GFP, -STAT3, -STAT5, -cyclin D1, -cyclin D2, and -γ-tubulin antibodies on 20 μg of protein from *GFP*$^+$ or *ICA512-CCF-GFP*$^+$ INS-1 cells cotransfected with *control* or *STAT3* hairpin for RNAi. Three days posttransfection, the cells were treated and stimulated as in Fig. 3C.

Materials and Methods

Culture and Transfection of INS-1 Cells. INS-1 cells (a gift of C. Wollheim, Geneva, Switzerland) were grown as described in ref. 21. In brief, 3 days posttransfection, cells were cultured for 18 h in serum-free media. On day 4, cells were incubated for 2 h in resting buffer (0 mM glucose, 5 mM KCl) or stimulated by either (*i*) 25 mM glucose; (*ii*) 25 mM glucose and 55 mM KCl; (*iii*) 25 mM glucose, 55 mM KCl, and 20 nM human growth hormone; (*iv*) 20 nM human growth hormone; (*v*) 20 nM human growth hormone plus 5 mM KCl. Next, cells were incubated for 4 h in RPMI-1640 with low (0.5%) FBS, 11 mM glucose, 25 mM Hepes, 1% penicillin–streptomycin, and 0.05 mM β-mercaptoethanol. In some instances, starting from the 18-h incubation in serum-free media until cells were harvested, the stimulation protocol was carried out in the presence of an insulin antibody (1:1000, Sigma) to neutralize released insulin.

Cell Extraction and Immunoblotting. INS-1 cells and pancreatic islets were harvested at 4°C in RIPA buffer [50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitor mixture (Sigma)] for total protein extraction. Insoluble material was removed by centrifugation. Aliquots of 20 μg of protein were separated by 8–10% SDS/PAGE and immunoblotted with the following antibodies: mouse monoclonal anti-ICA512 (23); anti-STAT5 (Santa Cruz); anti-γ-tubulin and anti-cyclin D1 (Neomarkers); anti-cyclin D2 (Abcam); rabbit anti-PY-STAT5 and anti-STAT3 (Cell Signaling); affinity-purified goat anti-GFP IgGs (Max Planck Institute for Molecular Cell Biology and Genetics). Chemiluminescence was developed with the Supersignal West Pico or Femto kits (Pierce) and detected with a LAS 3000 Bioimaging System (Fuji). Protein signals were quantified with the Image Gauge v3.45 software (Fuji).

RNA Interference. Two silencing hairpin DNA oligonucleotides for ICA512, STAT3, or STAT5b were cloned into the *pGENECLIP* vector (Promega) according to the manufacturer's instructions by using the following primers: *ICA512 oligo1*, 5′-TCTCGCGCCATCATTCGAAACAATTCAAGAGATTGTTTCGAATGATGGCGCCT-3′; *ICA512 oligo2*, 5′-TCTCGCAGTACAAGCAGATGTAATTCAAGAGATTACATCTGCTTGTACTGCCT-3′; *STAT3 oligo*1, 5′-TCTCGCGTGTGCAGGATCTAGAATTCAAGAGATTCTAGATCCTGCACCGCCT-3′, *STAT3 oligo2*, 5′-TCTCGAGGGTCTCGGAAATTTAATTCAAGAGATTAAATTTCCGAGACCCTCCT-3′, *STAT5b oligo1*, 5′-TCTCGAGGAGCTGCGTCTGATCAAAGTTCTCTTGATCAGACGCAGCTCCTCCT-3′; *STAT5b oligo2*, 5′-TCTCGGAAGCTGAACGTGCACATAAGTTCTCTATGTGCACGTTCAGCTTCCCT-3′. Four micrograms of either plasmid alone or in combination was transfected into INS-1 cells by electroporation as described in ref. 19. Cells were harvested 4 days after transfection and gene knockdown was verified by real-time PCR and Western blot as described in ref. 48.

PCR and Real-Time PCR. For quantification of *cyclin D1* and *ICA512* mRNAs, RNA was isolated with the Oligotex direct mRNA kit (Qiagen) according to the manufacturer's protocol. PolyA$^+$-enriched RNA was reverse transcribed with antisense primers specific for *ICA512* and β-actin as described in ref. 48. C*yclin D1* was reverse transcribed by using the following oligonucleotides: forward, 5′-ccgcacaacgcactttctttcca-3′; reverse, 5′-gatgtccacatctcggacgtc-3′. Real-time PCR was performed as described in ref. 48.

Partial Pancreatectomy. All studies involving animals were approved by the Institutional Animal Care and Use Committee of the University of Dresden and the Saxonian Government. *ICA512*−/− mice were provided by M.-S. Lee (Seoul, South Korea). Control and *ICA512*−/− mice were genotyped as described in ref. 23. Mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. The abdomen was opened through an upper midline incision. The spleen and the entire splenic portion of the pancreas were surgically removed, but the mesenteric pancreas between the portal vein and the duodenum was left intact. The remnant was defined as the pancreatic tissue within 1–2 mm of the common bile duct that extends from the duct to the first portion of the duodenum. This remnant is the upper portion of the head of the pancreas. This procedure resulted in a ≈75% pancreatectomy, confirmed by weighing the removed and remnant portions. Sham operations were performed by removing the spleen while leaving the pancreas intact. At the end of surgery, Alzet 1007D miniosmotic pumps (Alza) were implanted i.p. to deliver 50 $\mu g \cdot \mu l^{-1}$ BrdU (Sigma) in 50% DMSO at a rate of 0.5 $\mu l \cdot h^{-1}$ for 7 days. Blood glucose levels were

measured daily with a Glucotrend glucometer (Roche Diagnostics). The remnants and the sham-operated pancreata were harvested 7 days postsurgery after euthanasia of anesthetized animals.

Immunostaining. After fixation by intracardial perfusion with 4% paraformaldehyde, mouse pancreata were removed, further fixed overnight in 10% neutral formalin, and embedded in paraffin. Sections were cut at 5 μm. After dewaxing and microwave antigen retrieval, slides were briefly incubated with 0.2% Triton X-100 and 10% serum and then overnight at 4°C with guinea pig anti-insulin (Abcam) and mouse anti-BrdU (Roche Diagnostics) antibodies followed by Alexa^{568}-goat anti-guinea pig (Molecular Probes) and FITC-goat anti-mouse (Roche Diagnostics) antibodies. Nuclei were counterstained with DAPI. Confocal images of pancreatic sections from four sham-operated and nine partially pancreatectomized mice per group from three separate surgical series were collected. All BrdU$^+$ and insulin$^+$ cells (β-cells) from 50 islets per mouse were counted. Percentage of β-cell proliferation was calculated by dividing the number of BrdU$^+$ β-cells by the total number of β-cells. Ten images were used to calculate the total number of insulin$^-$ and BrdU$^+$/insulin$^-$ cells in each group of mice. Percentage of proliferation among non-β-cells was calculated by dividing the number of BrdU$^+$/insulin$^-$ cells by the total number of insulin$^-$ cells.

BrdU Labeling of INS-1 Cells. INS-1 cells transfected with *GFP* or *ICA512-CCF-GFP* were grown for 48 h on coverslips in six-well plates in RPMI-1640 containing 5% FBS, then made quiescent by incubation for 18 h in serum-free media. On day 4 postelectroporation, INS-1 cells were stimulated with 20 nM growth hormone for 20 min before being incubated with BrdU in RMPI-1640 for 8 h. After three washes in PBS, BrdU labeling was detected as described above. For [^{3}H]thymidine incorporation, cells in 35-mm wells were treated as described for BrdU staining until their stimulation with growth hormone was terminated. The cells were then cultured for various times in RPMI-1640 containing 10% FBS and 10 μCi of [^{3}H]thymidine per well.

Statistics and Graphics. Statistical analyses were performed by using the unpaired Student's t test. Results are presented as mean $\pm$ SE unless otherwise stated. A value of $P < 0.05$ was considered significant. Error bars show standard deviations from at least three independent experiments unless otherwise stated. Histograms were prepared with Microsoft Excel (Microsoft).

ACKNOWLEDGMENTS. We thank Antonello Pileggi, Luca Inverardi, and Camillo Ricordi for advice on islet procedures; Myung-Shik Lee for ICA512$^{-/-}$ mice; Claes Wollheim for INS-1 cells; Anke Altkrüger, Anja Lohmann, Melanie Jäger, and Katja Pfriem for assistance; Ron Dirkx for reading the manuscript; and Laurel Rohde for manuscript editing. This work was supported by grants from the Juvenile Diabetes Research Foundation (1-2004-2567), the European Foundation for the Study of Diabetes, the German Research Foundation (SFB655), and the German Ministry for Education and Research (NBL-3) (to M.S.), and by a MedDrive grant from the Medical School at the Dresden University of Technology and a travel fellowship from the European Foundation for the Study of Diabetes (to S.K.).

1. Rhodes CJ (2005) Type 2 diabetes-a matter of beta-cell life and death? *Science* 307:380–384.
2. Kloppel G, Lohr M, Habich K, Oberholzer M, Heitz PU (1985) Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. *Surv Synth Pathol Res* 4:110–125.
3. Heit JJ, Karnik SK, Kim SK (2005) Intrinsic regulators of pancreatic beta-cell proliferation. *Annu Rev Cell Dev Biol* 22:311–338.
4. Bouwens L, Rooman I (2005) Regulation of pancreatic beta-cell mass. *Physiol Rev* 85:1255–1270.
5. Finegood DT, Scaglia L, Bonner-Weir S (1995) Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. *Diabetes* 44:249–256.
6. Bonner-Weir S (2000) Perspective: Postnatal pancreatic beta cell growth. *Endocrinology* 141:1926–1929.
7. Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429:41–46.
8. Bouwens L (1998) Transdifferentiation versus stem cell hypothesis for the regeneration of islet beta-cells in the pancreas. *Microsc Res Tech* 43:332–336.
9. Nielsen JH, *et al.* (2001) Regulation of beta-cell mass by hormones and growth factors. *Diabetes* 50:25–29.
10. Seufert J, Kieffer TJ, Habener JF (1999) Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. *Proc Natl Acad Sci USA* 96:674–679.
11. Saxena NK, Vertino PM, Anania FA, Sharma D (2007) Leptin-induced growth stimulation of breast cancer cells involves recruitment of histone acetyltransferases and mediator complex to CYCLIN D1 promoter via activation of Stat3. *J Biol Chem* 282:13316–13325.
12. Friedrichsen BN, *et al.* (2003) Signal transducer and activator of transcription 5 activation is sufficient to drive transcriptional induction of cyclin d2 gene and proliferation of rat pancreatic β-cells. *Mol Endocrinol* 17:945–958.
13. Brelje TC, Stout LE, Bhagroo NV, Sorenson RL (2004) Distinctive roles for prolactin and growth hormone in the activation of signal transducer and activator of transcription 5 in pancreatic islets of langerhans. *Endocrinology* 145:4162–4175.
14. Galsgaard ED, *et al.* (1996) Identification of a growth hormone-responsive STAT5-binding element in the rat insulin 1 gene. *Mol Endocrinol* 10:652–660.
15. Galsgaard ED, Nielsen JH, Moldrup A (1999) Regulation of prolactin receptor (PRLR) gene expression in insulin-producing cells. Prolactin and growth hormone activate one of the rat prlr gene promoters via STAT5a and STAT5b. *J Biol Chem* 274:18686–18692.
16. Ihle JN (2001) The Stat family in cytokine signaling. *Curr Opin Cell Biol* 13:211–217.
17. Levy DE, Darnell JE, Jr (2002) Stats: Transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 3:651–662.
18. Teglund S, *et al.* (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* 93:841–850.
19. Trajkovski M, *et al.* (2004) Nuclear translocation of an ICA512 cytosolic fragment couples granule exocytosis and insulin expression in β-cells. *J Cell Biol* 167:1063–1074.
20. Solimena M, *et al.* (1996) ICA 512, an autoantigen of type I diabetes, is an intrinsic membrane protein of neurosecretory granules. *EMBO J* 15:2102–2114.
21. Ort T, *et al.* (2001) Dephosphorylation of beta2-syntrophin and Ca2+/mu-calpain-mediated cleavage of ICA512 upon stimulation of insulin secretion. *EMBO J* 20:4013–4023.
22. Mziaut H, *et al.* (2006) Synergy of glucose and growth hormone signalling in islet cells through ICA512 and STAT5. *Nat Cell Biol* 8:435–445.
23. Hugl SR, White MF, Rhodes CJ (1998) Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent. Synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *J Biol Chem* 273:17771–17779.
24. Gahr S, *et al.* (2002) Hepatocyte growth factor stimulates proliferation of pancreatic beta-cells particularly in the presence of subphysiological glucose concentrations. *J Mol Endocrinol* 28:99–110.
25. Cousin SP, *et al.* (1999) Stimulation of pancreatic beta-cell proliferation by growth hormone is glucose-dependent: signal transduction via janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) with no cross-talk to insulin receptor substrate-mediated mitogenic signalling. *Biochem J* 344(Pt 3):649–658.
26. Bonner-Weir S, Trent DF, Weir GC (1983) Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. *J Clin Invest* 71:1544–1553.
27. Leahy JL, Bonner-Weir S, Weir GC (1988) Minimal chronic hyperglycemia is a critical determinant of impaired insulin secretion after an incomplete pancreatectomy. *J Clin Invest* 81:1407–1414.
28. Bonner-Weir S, Sharma A (2002) Pancreatic stem cells. *J Pathol* 197:519–526.
29. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE (1993) A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. *Diabetes* 42:1715–1720.
30. Saeki K, *et al.* (2002) Targeted disruption of the protein tyrosine phosphatase-like molecule IA-2 results in alterations in glucose tolerance tests and insulin secretion. *Diabetes* 51:1842–1850.
31. Sekine N, Wollheim CB, Fujita T (1998) GH signalling in pancreatic beta-cells. *Endocr J* 45(Suppl):S33–S40.
32. Kushner JA, *et al.* (2005) Cyclins D2 and D1 are essential for postnatal pancreatic beta-cell growth. *Mol Cell Biol* 25:3752–3762.
33. Friedrichsen BN, Galsgaard ED, Nielsen JH, Moldrup A (2001) Growth hormone- and prolactin-induced proliferation of insulinoma cells, INS-1, depends on activation of STAT5 (signal transducer and activator of transcription 5). *Mol Endocrinol* 15:136–148.
34. Xu G, Stoffers DA, Habener JF, Bonner-Weir S (1999) Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48:2270–2276.
35. Suarez-Pinzon WL, Yan Y, Power R, Brand SJ, Rabinovitch A (2005) Combination therapy with epidermal growth factor and gastrin increases beta-cell mass and reverses hyperglycemia in diabetic NOD mice. *Diabetes* 54:2596–2601.
36. Gupta RK, *et al.* (2007) Expansion of adult beta-cell mass in response to increased metabolic demand is dependent on HNF-4alpha. *Genes Dev* 21:756–769.
37. Heit JJ, *et al.* (2006) Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. *Nature* 443:345–349.
38. Rulifson IC, *et al.* (2007) Wnt signaling regulates pancreatic beta cell proliferation. *Proc Natl Acad Sci USA* 104:6247–6252.
39. Nikolova G, *et al.* (2006) The vascular basement membrane: a niche for insulin gene expression and Beta cell proliferation. *Dev Cell* 10:397–405.
40. Terauchi Y, *et al.* (2007) Glucokinase and IRS-2 are required for compensatory beta cell hyperplasia in response to high-fat diet-induced insulin resistance. *J Clin Invest* 117:246–257.
41. Weir GC, Bonner-Weir S (2007) A dominant role for glucose in beta cell compensation of insulin resistance. *J Clin Invest* 117:81–83.
42. Okada T, *et al.* (2007) From the Cover: Insulin receptors in beta-cells are critical for islet compensatory growth response to insulin resistance. *Proc Natl Acad Sci USA* 104:8977–8982.
43. Park S, *et al.* (2006) Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. *J Biol Chem* 281:1159–1168.
44. Tsukiyama S, *et al.* (2006) Transduction of exogenous constitutively activated Stat3 into dispersed islets induces proliferation of rat pancreatic beta-cells. *Tissue Eng* 12:131–140.
45. Sherr CJ (1995) D-type cyclins. *Trends Biochem Sci* 20:187–190.
46. Georgia S, Bhushan A (2004) Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest* 114:963–968.
47. Cozar-Castellano I, Takane KK, Bottino R, Balamurugan AN, Stewart AF (2004) Induction of beta-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1. *Diabetes* 53:149–159.
48. Knoch KP, *et al.* (2004) Polypyrimidine tract-binding protein promotes insulin secretory granule biogenesis. *Nat Cell Biol* 6:207–214.