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Deletion of *Cdkn1b* ameliorates hyperglycemia by maintaining compensatory hyperinsulinemia in diabetic mice

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The protein p27^{Kip1} regulates cell cycle progression in mammals by inhibiting the activity of cyclin-dependent kinases (CDKs). Here we show that p27^{Kip1} progressively accumulates in the nucleus of pancreatic beta cells in mice that lack either insulin receptor substrate 2 (*Irs2*$^{-/-}$) or the long form of the leptin receptor (*Lepr*$^{-/-}$ or *db/db*). Deletion of the gene encoding p27^{Kip1} (*Cdkn1b*) ameliorated hyperglycemia in these animal models of type 2 diabetes mellitus by increasing islet mass and maintaining compensatory hyperinsulinemia, effects that were attributable predominantly to stimulation of pancreatic beta-cell proliferation. Thus, p27^{Kip1} contributes to beta-cell failure during the development of type 2 diabetes in *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice and represents a potential new target for the treatment of this condition.

Type 2 diabetes mellitus is characterized by insulin resistance and dysfunction of pancreatic beta cells. The mass of pancreatic islets is also reduced in individuals with this condition[1–4], suggesting that beta-cell growth is essential to compensate for peripheral insulin resistance. Islet mass is determined by both islet density and islet size, the latter of which is a function of the number and size of the component cells. Cell number is determined by the balance between cell proliferation and death. Although the regulation of islet mass is complex, recent studies have implicated a signaling pathway in this process that is triggered by insulin or insulin-like growth factor-1 (IGF-1)[5–7]. Irs2-deficient (*Irs2*$^{-/-}$) mice do not maintain islet mass in the face of hepatic insulin resistance[8,9]. Furthermore, Akt, which is activated by signaling through Irs2 and phosphatidylinositol-3-kinase (PI3K), promotes the growth of islets in mice[10–12]. This effect of Akt is mediated by the transcriptional regulator Foxo1 (refs. 13–15). The downstream effectors that are involved in the regulation of islet mass have yet to be isolated.

Progression of the cell cycle in mammals is governed by various complexes of cyclins and CDKs and by their inhibitors (CKIs)[16]. Mice that lack Cdk4 develop insulin-dependent diabetes as a result of a reduction in islet mass[17]. Little is known, however, about whether disturbance of the cell cycle in beta cells actually contributes to the pathogenesis of diabetes and whether manipulation of the cell cycle has the potential to restore islet mass during the development of this condition. The CKI p27^{Kip1} (p27), through its inhibition of CDK activity, is important for cell cycle regulation[18]. Akt modulates p27 function by affecting both its mRNA abundance through the Foxo family of transcriptional regulators[13,19] and its subcellular localization by direct phosphorylation[20–23]. These observations prompted us to examine the possible role of p27 in beta cells during the development of type 2 diabetes.

Here we show that, as compared with wild-type mice, the overall abundance and nuclear accumulation of p27 are both increased in the beta cells of *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice. Furthermore, deletion of *Cdkn1b* in the *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mouse models of type 2 diabetes prevented the development of overt hyperglycemia and increased both islet mass and the serum concentration of insulin. Our findings indicate a potential new strategy for the treatment of diabetes based on the promotion of cell cycle progression in beta cells.

RESULTS

Accumulation of p27 in beta cells of diabetic mice

Given that the abundance and subcellular localization of p27 are modulated by Akt, we examined the expression of p27 in islets of *Irs2*$^{-/-}$ mice. Immunostaining of pancreatic sections from 4-, 8- and 18-week-old *Irs2*$^{-/-}$ mice with antibodies specific to p27 showed a progressive increase in the amount of p27 in islets (**Fig. 1a**). We next examined p27 expression in *Lepr*$^{-/-}$ mice. These mice develop obesity because of hyperphagia, decreased energy expenditure and preferential partitioning of calories into adipose tissue triglycerides[24–26]. Although the beta cells of *Lepr*$^{-/-}$ mice initially grow to compensate for the increased insulin resistance, they eventually do not during progression of hyperglycemia, with a consequent marked decrease in islet mass[27]. Immunostaining showed that the amount of p27 increased progressively in the islets of

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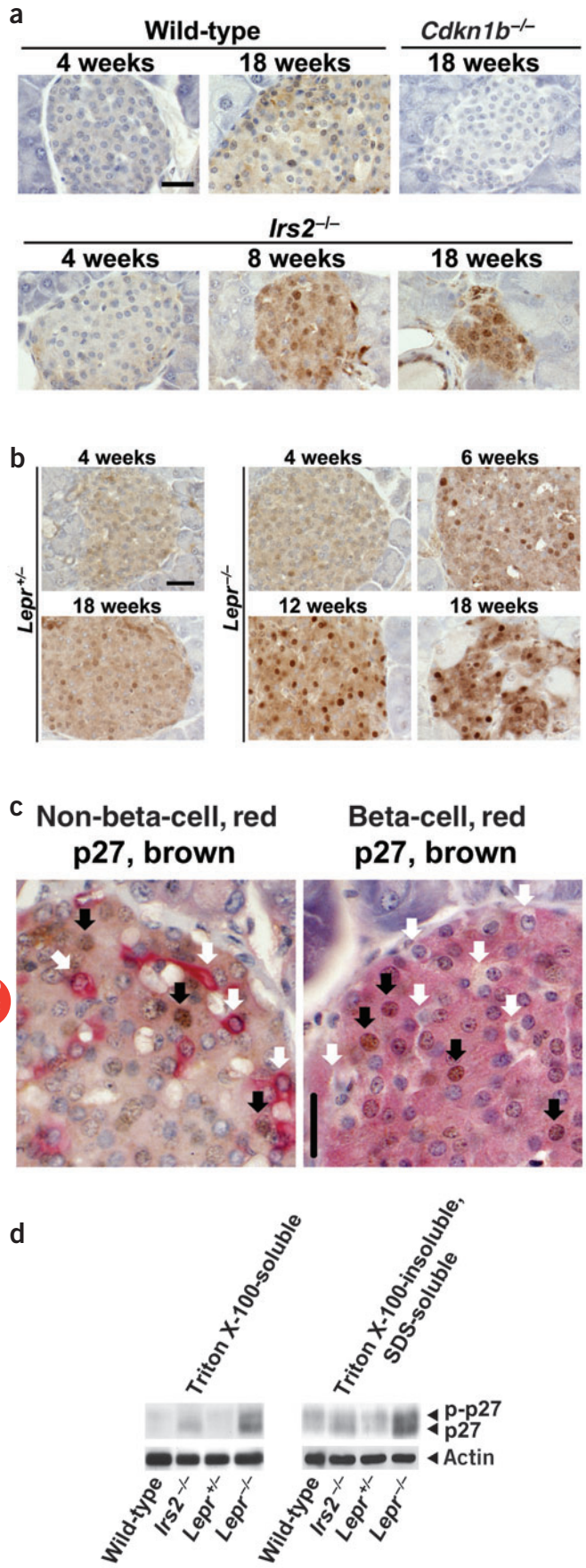

Figure 1 Accumulation of p27 in beta cells of diabetic mice. (**a**) Pancreatic sections from wild-type, *Irs2*$^{-/-}$ and *Cdkn1b*$^{-/-}$ mice at indicated ages were immunostained with antibodies to p27 and counterstained with hematoxylin. Scale bar, 25 μm. (**b**) Pancreatic sections from *Lepr*$^{-/-}$ and *Lepr*$^{+/-}$ mice at indicated ages were immunostained with antibodies to p27. Scale bar, 25 μm. (**c**) Pancreatic sections from a 12-week-old *Lepr*$^{-/-}$ mouse immunostained with antibodies to p27 and antibodies to either insulin (beta-cell marker) or a non-beta-cell marker (glucagon, somatostatin, pancreatic polypeptide). Black arrows indicate p27-stained nuclei; white arrows indicate non-beta cells. Scale bar, 25 μm. (**d**) Islets were isolated from 8-week-old wild-type, *Irs2*$^{-/-}$, *Lepr*$^{+/-}$ and *Lepr*$^{-/-}$ mice and lysed, and the indicated fractions were immunoblotted with antibodies to p27 and to actin. The positions of nonphosphorylated and phosphorylated (p-p27) forms of p27 are indicated.

Lepr$^{-/-}$ mice during the development of obesity and diabetes, with most of the protein being localized in cell nuclei (**Fig. 1b**).

Immunostaining with antibodies to p27 and either those to insulin (beta-cell marker) or those to non-beta-cell markers (glucagon, somatostatin and pancreatic polypeptide) showed that p27 accumulated specifically in the beta cells of *Lepr*$^{-/-}$ mouse islets (**Fig. 1c**). We also examined the expression of other CKIs, including p16^{INK4a}, p21^{Cip1} and p57^{Kip2}, in the islets of *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice at 8 weeks of age. Immunoblot analysis showed similar low amounts of p21^{Cip1} expression in the islets of these mice and in those of control mice (see **Supplementary Fig. 1** online). Neither p16^{INK4a} nor p57^{Kip2} was detected in islets from *Irs2*$^{-/-}$, *Lepr*$^{-/-}$ and control mice (data not shown).

Immunoblot analysis confirmed that the abundance of p27 in either cytoplasm-enriched (Triton X-100-soluble) or nuclear (Triton X-100-insoluble, sodium dodecylsulfate–soluble) fractions of islets isolated from 8-week-old *Irs2*$^{-/-}$ mice was 1.9 times that in the corresponding fractions of wild-type islets (**Fig. 1d**). The amount of p27 in the islets of 8-week-old *Lepr*$^{-/-}$ mice was also increased by factors of 2.5 in the cytoplasm-enriched fraction and 4.2 in the nuclear fraction as compared with that in the corresponding fractions of *Lepr*$^{+/-}$ islets (**Fig. 1d**). Immunoblot analysis detected p27 as two distinct bands, the upper of which comigrated with phosphorylated p27 (**Supplementary Fig. 2**). The upper band was predominant in the islets from control mice, whereas the lower band was predominant in the islets from *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice (**Fig. 1d**). Thus, these results suggest that the nonphosphorylated form of p27 accumulates in the islets of diabetic mice.

Overexpression of p27 in beta cells induces diabetes

To examine whether increased expression of p27 in beta cells might cause diabetes, we generated mice that express the human gene that encodes p27 (*CDKN1B*) under the control of the promoter of the rat insulin gene (*RIP-CDKN1B* mice). Human p27 was highly expressed in the beta-cells of the transgenic mice as shown by immunostaining with antibodies specific to the human protein (**Fig. 2a**). Immunoblot analysis with antibodies that recognize both human and mouse p27 indicated that the total amount of p27 in islets of *RIP-CDKN1B* mice was 6.2 times that in the islets of control littermates (**Fig. 2b**).

At as early as 4 weeks of age, *RIP-CDKN1B* mice had hyperglycemia, which subsequently progressed to severe diabetes (**Fig. 2c**). The serum concentration of insulin decreased in *RIP-CDKN1B* mice during development of diabetes (**Fig. 2d**). Immunostaining with antibodies to insulin and to glucagon showed that the number of beta-cells was reduced in the islets of 8-week-old *RIP-CDKN1B* mice as compared with wild-type controls (**Fig. 2e**). In addition, the total islet mass (**Fig. 2f**) and islet density (number of islets per pancreas; **Fig. 2g**) of 8-week-old *RIP-CDKN1B* mice were both significantly smaller than those of littermate controls, whereas the area of individual beta cells did not differ between the two genoty-

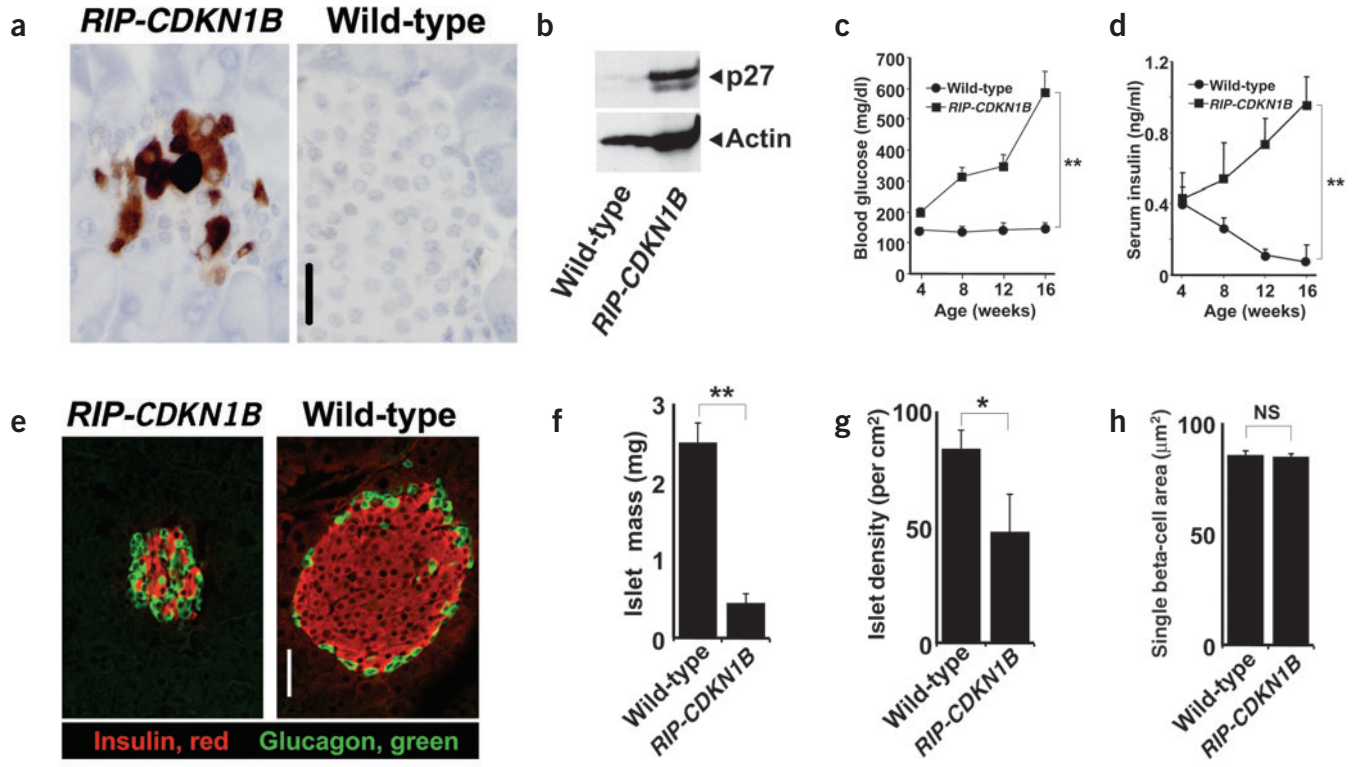

Figure 2 Induction of diabetes by overexpression of p27 in mouse beta cells. (**a**) Pancreatic sections from 8-week-old wild-type and RIP-CDKN1B mice were immunostained with antibodies to human p27 and were counterstained with hematoxylin. Scale bar, 25 μm. (**b**) Islets were purified from 8-week-old wild-type and *RIP-CDKN1B* mice and were lysed. Lysates were immunoblotted with antibodies to p27 (human and mouse) and to actin. (**c**) Blood glucose concentrations of fed mice at the indicated ages. (**d**) Serum insulin concentrations of fed mice at the indicated ages. (**e**) Pancreatic sections from 8-week-old mice of the indicated genotypes were stained with antibodies to insulin and to glucagon. Scale bar, 50 μm. (**f**,**g**) Random sections of the entire pancreas from 8-week-old mice of the indicated genotypes were stained with hematoxylin and eosin, and the number and size of all islets (more than five cells) were measured to determine (**f**) islet mass and (**g**) islet density. (**h**) The size of individual beta cells was estimated by measuring the area of at least 2,000 beta cells from mice of the indicated genotypes. $*P < 0.05$; $**P < 0.01$; n.s., not significant.

pes (**Fig. 2h**). Although we detected proliferating-cell nuclear antigen (Pcna) in the beta cells of 8-week-old wild-type mice, it was not apparent in the beta cells of *RIP-CDKN1B* mice (**Supplementary Fig. 3** online). Increased expression of p27 in beta cells thus induced severe diabetes as a result of inhibition of beta-cell proliferation. The increased abundance and nuclear accumulation of p27 that was apparent in the beta cells of *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice are therefore a likely cause of the beta-cell failure in these mouse models of type 2 diabetes.

Deletion of *Cdkn1b* ameliorates hyperglycemia in diabetic mice

We next made use of *Cdkn1b*$^{-/-}$ mice to characterize further the effects of endogenous p27 during the development of diabetes. We generated mice with a combined deficiency of Irs2 and p27 by interbreeding *Irs2*$^{+/-}$ *Cdkn1b*$^{+/-}$ mice. As shown previously, at about 10 weeks of age *Irs2*$^{-/-}$ mice develop dyslipidemia and hyperglycemia that subsequently progress to diabetes[8,9] (**Table 1** and **Fig. 3a**). The blood glucose concentrations of *Irs2*$^{-/-}$*Cdkn1b*$^{+/-}$ and *Irs2*$^{-/-}$*Cdkn1b*$^{-/-}$ mice, however, remained near normal up to 18 weeks of age (**Fig. 3a**). Whereas the fasting serum insulin concentration of *Irs2*$^{-/-}$ mice was similar to that of wild-type mice at 18 weeks of age, those of *Irs2*$^{-/-}$*Cdkn1b*$^{+/-}$ and *Irs2*$^{-/-}$*Cdkn1b*$^{-/-}$ mice were significantly increased (**Fig. 3b**), suggesting that deletion of *Cdkn1b* promotes compensatory beta-cell function in *Irs2*$^{-/-}$ mice.

We also examined the effects of *Cdkn1b* deletion in *Lepr*$^{-/-}$ mice. Mice with a combined deficiency of the leptin receptor and p27 were generated by interbreeding *Lepr*$^{+/-}$*Cdkn1b*$^{+/-}$ mice on a mixed C57BL/6J × C57BL/KsJ background. The *Lepr*$^{-/-}$, *Lepr*$^{-/-}$*Cdkn1b*$^{+/-}$ and *Lepr*$^{-/-}$ *Cdkn1b*$^{-/-}$ mice showed similar obesity, hyperphagia, hypothermia and hyperlipidemia (**Table 1**). Hyperglycemia developed more slowly and to a reduced extent in *Lepr*$^{-/-}$*Cdkn1b*$^{+/-}$ mice than in *Lepr*$^{-/-}$ mice, however (**Fig. 3c**). Furthermore, in contrast to *Irs2*$^{-/-}$ mice, deletion of both *Cdkn1b* alleles completely normalized hyperglycemia in *Lepr*$^{-/-}$ mice; the effect of *Cdkn1b* deletion in *Lepr*$^{-/-}$ mice was thus dependent on gene dosage. To reduce genetic variation, we backcrossed the disrupted *Cdkn1b* allele into the C57BL/KsJ background for five generations. Similar results were obtained with *Lepr*$^{-/-}$*Cdkn1b*$^{+/-}$ and *Lepr*$^{-/-}$ *Cdkn1b*$^{-/-}$ mice with the C57BL/KsJ background (**Fig. 3c**). Whereas the extent of hyperinsulinemia declined in *Lepr*$^{-/-}$ mice, it increased significantly in *Lepr*$^{-/-}$*Cdkn1b*$^{+/-}$ and *Lepr*$^{-/-}$*Cdkn1b*$^{-/-}$ mice (**Fig. 3d**).

Deletion of *Cdkn1b* increases islet mass in diabetic mice

Given that *Cdkn1b* deletion resulted in an increase in the serum insulin concentration in *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice, the amelioration of hyperglycemia that was observed in the double-knockout mice is probably to the result of improved beta-cell function rather than reduced insulin resistance. Therefore, we first measured islet mass. Islet mass was increased only slightly in *Cdkn1b*$^{-/-}$ mice as compared with that in wild-type mice (**Fig. 4a**). Islet mass was significantly decreased in *Irs2*$^{-/-}$ mice and increased in *Lepr*$^{-/-}$ mice as compared with their respective controls at 12 weeks of age. Deletion of *Cdkn1b* resulted in a significant increase in islet mass in both *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice.

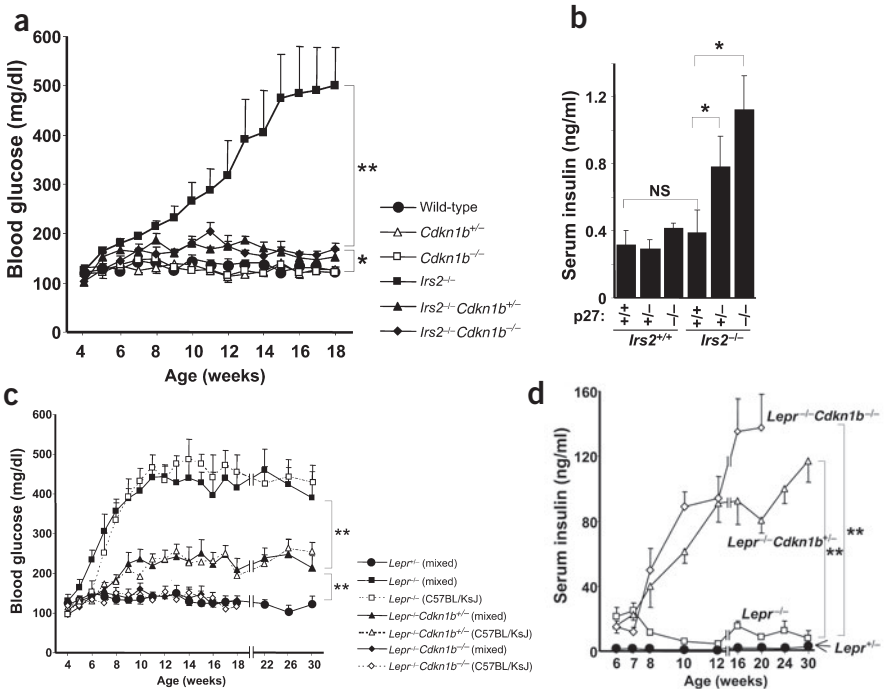

Figure 3 Improvement in glucose metabolism induced by *Cdkn1b* disruption in *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice. (**a**) Glucose concentrations in blood derived from the tail vein of fed mice at the indicated ages. (**b**) Serum insulin concentrations in 18-week-old mice of the indicated genotypes that were in the fasting state. (**c**) Blood glucose concentrations of fed mice at the indicated ages. (**d**) Serum insulin concentrations in fed mice of the indicated genotypes (on the C57BL/KsJ background) and ages. $^{*}P < 0.05$; $^{**}P < 0.01$; n.s., not significant.

Islet mass is determined by both islet density and islet size. Whereas the islet density of 12-week-old *Irs2*$^{-/-}$ mice was decreased as compared with that of control mice, islet density of *Lepr*$^{-/-}$ mice was increased; the islet density in mice of neither of these genotypes was affected by *Cdkn1b* deletion, however (**Fig. 4b**,**c**). In contrast, islet size in *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice, which was decreased and increased, respectively, as compared with that in control mice, was markedly increased by *Cdkn1b* deletion (**Fig. 4c**,**d**), suggesting that p27 regulates islet growth.

Deletion of *Cdkn1b* increased the number of beta cells rather than that of alpha cells in mice of both genotypes (**Fig. 4d**). The size of individual beta cells, which was estimated by measurement of beta-cell area, was reduced in *Irs2*$^{-/-}$ mice and increased in *Lepr*$^{-/-}$ mice relative to that in control mice, but this parameter was not affected by *Cdkn1b* deletion (**Fig. 4e**). We also examined the extent of apoptosis and proliferation in islets with the terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) assay and immunostaining with antibodies to Pcna, respectively. Apoptotic nuclei were rarely observed in the islets of *Lepr*$^{+/-}$ and *Lepr*$^{-/-}$ mice, and their abundance was not affected by *Cdkn1b* deletion (**Supplementary Fig. 4** online). To provide further support for this conclusion, we examined the effects of p27 overexpression in mouse Min6 insulinoma cells. Although overexpression of p27 reduced the rate of increase in cell number during culture, it did not affect the proportion of apoptotic cells as detected by DNA fragmentation or cleavage of caspase-3 during incubation with or without serum or even with tunicamycin (**Supplementary Fig. 5** online).

In contrast, the number of Pcna-positive beta cells was markedly increased by *Cdkn1b* deletion in the islets of *Irs2*$^{-/-}$ (**Supplementary Fig. 6** online) and *Lepr*$^{-/-}$ (**Fig. 4f**,**g**) mice. These results thus indicate that p27 may function as a negative regulator of beta-cell proliferation during the development of diabetes in *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice.

Regulation of p27 by IGF-1 and leptin in beta cells

Irs2 contributes to the growth effects of IGF-1, although this contribution has not been conclusively shown in beta cells[6,7,28]. To determine whether this signaling pathway also affects p27, we stimulated dissociated islet cells from wild-type or *Irs2*$^{-/-}$ mice with IGF-1 and then immunostained them for p27 and insulin. Whereas p27 was localized in both the nucleus and cytoplasm of unstimulated beta cells of each genotype, IGF-1 induced the nuclear export of p27 in wild-type beta cells but not in *Irs2*$^{-/-}$ beta cells (**Fig. 5a**). These observations indicate that IGF-1-Irs2 signaling promotes the exclusion of p27 from the nucleus in beta cells.

We next examined Irs2 signaling in islets from 8-week-old *Lepr*$^{-/-}$ mice. We prepared Irs2 immunoprecipitates from the islets and subjected them to immunoblot analysis with antibodies to Irs2 and to the p85 regulatory subunit of PI3K. The amounts of Irs2 were similar in islets from *Lepr*$^{-/-}$ and control *Lepr*$^{+/-}$ mice, although the Irs2 band from *Lepr*$^{-/-}$ mice migrated more slowly (**Fig. 5c**). The amount of p85 that was associated with Irs2 in islets from *Lepr*$^{-/-}$ mice was, however, three times that in *Lepr*$^{+/-}$ islets (**Fig. 5b**), indicating that Irs2 signaling may be enhanced in *Lepr*$^{-/-}$ islets.

The activity of Akt in islets from *Irs2*$^{-/-}$ mice was greatly reduced as compared with that in wild-type islets (**Fig. 5c**). Despite the increased association of p85 with Irs2 in islets from *Lepr*$^{-/-}$ mice, the activity of Akt was also reduced in the islets of these mice. Deletion of *Cdkn1b* had no effect on Akt activity in the islets from either type of diabetic mouse (**Fig. 5c**). We also examined the effect of leptin signaling on Akt activity in Min6 insulinoma cells, which proliferate in response to leptin[29]. The activity of Akt in these cells was increased by stimulation with leptin or IGF-1 (**Supplementary Fig. 7** online). Finally, we examined the effect of leptin on the subcellular localization of p27 in dissociated islet cells from wild-type and *Lepr*$^{-/-}$ mice. Whereas leptin induced the exclusion of p27 from the nucleus of wild-type beta cells, it had no such effect in *Lepr*$^{-/-}$ beta-cells (**Fig. 5d**). These data indicate that leptin signaling, at least in part through the activation of Akt, may regulate the subcellular localization of p27.

DISCUSSION

We have shown that the abundance and nuclear accumulation of p27 are increased in the beta cells of *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice, two animal models of human type 2 diabetes. Furthermore, overexpression of human p27 specifically in the beta cells of wild-type mice resulted in severe diabetes. Conversely, deletion of *Cdkn1b* increased islet mass and the serum insulin concentration in both *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice and prevented the development of overt hyperglycemia. Our results thus indicate that an accumulation of p27 in beta cells may contribute to the pathogenesis of type 2 diabetes in *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice.

The increase in islet mass that was induced by *Cdkn1b* deletion in these diabetic mice was attributable to an increase in beta-cell number without a change in either islet density or the size of individual beta cells. Furthermore, our data indicate that the increase in beta-cell number may have been to the result of an increase in cell proliferation without a

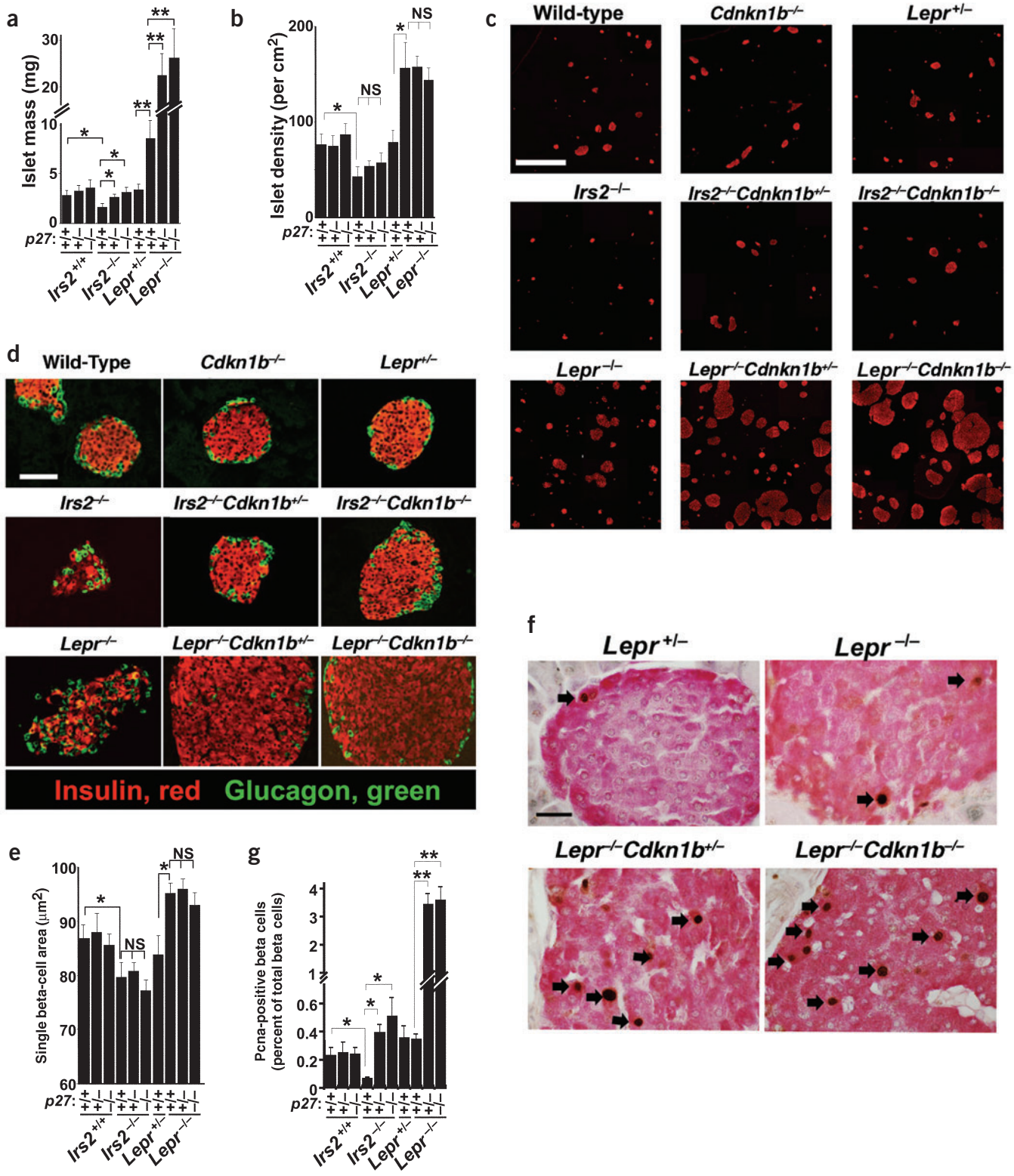

Figure 4 Effects of *Cdkn1b* deletion on islet characteristics. (**a**,**b**) Random sections of the entire pancreas from 12-week-old mice of the indicated genotypes were stained with hematoxylin-eosin, and the number and size of all islets (more than five cells) were measured to determine (**a**) islet mass and (**b**) islet density. (**c**) Pancreatic sections from mice of the indicated genotypes were stained with antibodies to insulin. Scale bar, 1 mm. (**d**) Pancreatic sections from mice of the indicated genotypes were stained with antibodies to insulin and to glucagon. Scale bar, 50 μm. (**e**) The size of individual beta cells was estimated by measuring the area of at least 2,000 beta cells from mice of the indicated genotypes. (**f**) Pancreatic sections from mice of the indicated genotypes were stained with antibodies to Pcna (brown) and to insulin (red) and then were counterstained with hematoxylin. Arrowheads indicate proliferating cells. Scale bar, 25 μm. (**g**) The number of cells that are positive for both Pcna and insulin were quantified as a percentage of the total number of insulin-positive cells in the sections. $^{*}P < 0.05$; $^{**}P < 0.01$; n.s., not significant.

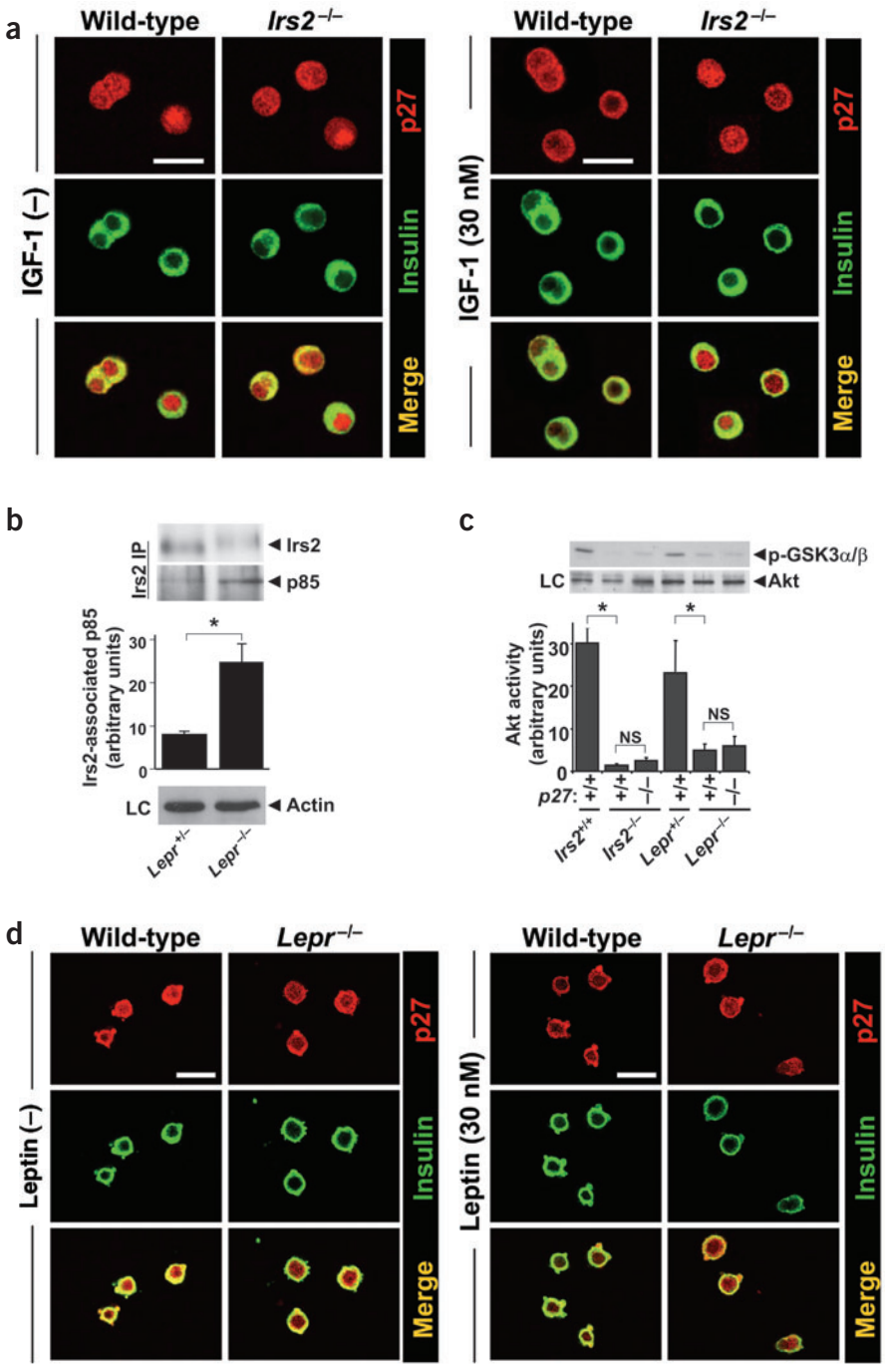

Figure 5 IGF-1 and leptin signaling promote nuclear exclusion of p27 in beta cells. (**a**) Dissociated beta cells from female wild-type and *Irs2*$^{-/-}$ mice were deprived of serum and were incubated in the absence or presence of IGF-1. The cells were then fixed and immunostained with antibodies to p27 and to insulin. Scale bars, 15 μm. (**b**) Lysates (100 μg of protein) prepared from islets of 8-week-old *Lepr*$^{-/-}$ and *Lepr*$^{+/-}$ mice were subjected to immunoprecipitation (IP) with antibodies to Irs2 and were immunoblotted with antibodies to Irs2 or to p85. As a loading control, lysates were immunoblotted (IB) with antibodies to actin. The amount of p85 associated with Irs2 was quantified. (**c**) Lysates prepared from islets of 8-week-old mice of the indicated genotypes were assayed for Akt activity with an immunocomplex kinase assay. The reaction mixture was immunoblotted with antibodies to phosphorylated GSK3α/β (upper panels). As a loading control, lysates were immunoblotted with antibodies to Akt (middle panels). The immunoblot band intensities were quantified (lower panel). (**d**) Dissociated beta cells from wild-type or *Lepr*$^{-/-}$ mice were analyzed as in a with the exception that they were stimulated with leptin instead of IGF-1. Scale bars, 20 μm. $^*P < 0.05$; n.s., not significant.

change in cell survival. These observations are consistent with the role of p27 as an inhibitor of cell cycle progression at G1 phase.

The PI3K-Akt signaling pathway negatively regulates several CKIs[30]. In mammalian cells, Akt both promotes the nuclear exclusion of p27 by direct phosphorylation[20–23], resulting in its degradation by the ubiquitin-proteasome pathway[31], and downregulates p27 expression by direct phosphorylation of Foxo1 (refs. 13,19). Although we did not detect an increase in the amount of *Cdkn1b* mRNA in the islets of *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice (**Supplementary Fig. 8** online), we cannot rule out the existence of a Foxo1-dependent mechanism of regulation of p27 in beta cells, given that deletion of *Foxo1* also rescues the diabetic phenotype in *Irs2*$^{-/-}$ mice[15]. In addition, Foxo1-dependent regulation of the subcellular localization of p27 was recently demonstrated in granulosa cells[32].

Dysregulation of Akt signaling might contribute to the nuclear accumulation of p27 that is apparent in the beta cells of *Lepr*$^{-/-}$ mice. The observation that the amount of p85 associated with Irs2 was increased in the islets of *Lepr*$^{-/-}$ mice indicates that Irs2 signaling may be enhanced. The activity of Akt was similarly decreased, however, in islets from *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice as compared with that in control islets. We also showed that leptin promotes the nuclear exclusion of p27 in wild-type beta cells, with this effect possibly being mediated by Akt activation. The interruption of this signaling pathway by the defect in the leptin receptor in *Lepr*$^{-/-}$ mice may explain, at least in part, the decreased Akt activity in the islets of these mice.

The localization of p27 in the nucleus is required for it to exert its CKI activity. The PI3K-Akt signaling pathway regulates the nuclear abundance and localization of p27 through phosphorylation on Ser10, Thr157, Thr187 and Thr198 (refs. 20–23). In addition, human kinase-interacting stathmin (hKIS) phosphorylates p27 on Ser10, thereby inducing its nuclear exclusion by promoting its interaction with Crm1 (refs. 33,34), a mediator of nuclear export. The cyclin E–Cdk2 complex phosphorylates p27 on Thr187, triggering its degradation in the nucleus[31,35–37]. The p90 ribosomal protein S6 kinase (p90^{Rsk}) induces the cytoplasmic localization of p27 through direct phosphorylation of Thr198 and its consequent binding to 14-3-3 (ref. 38). It is therefore possible that an abnormality in any of these signaling events is responsible for the increased nuclear abundance and localization of p27 in the beta cells of *Irs2*$^{-/-}$ and *Lepr*$^{-/-}$ mice.

Although Akt activity in islets was similar in the two types of diabetic mice, the abundance and nuclear accumulation of p27 were greater in the beta cells of *Lepr*$^{-/-}$ mice than in those of

Table 1 Characteristics of mice of various *p27*, *Irs2* and *Lepr* genotypes

	Body weight at 18 weeks (g)	Visceral fat at 18 weeks (g)	Food intake at 9 weeks (g/d)	Rectal temperature at 10 weeks (°C)	Serum triglyceride at 10 weeks (mg/dl)	Serum cholesterol at 10 weeks (mg/dl)	Serum FFA at 10 weeks (mg/dl)
Wild-type	31.9 ± 1.3	1,057.0 ± 82.3	4.2 ± 0.7	37.1 ± 0.2	88.2 ± 5.9	200.2 ± 16.2	1.05 ± 0.13
$p27^{+/-}$	35.8 ± 0.9[a]	1,163.5 ± 78.3	4.1 ± 0.3	37.0 ± 0.1	80.2 ± 6.7	190.3 ± 18.5	1.01 ± 0.14
$p27^{-/-}$	39.2 ± 1.2[a]	1,210.9 ± 94.3	4.2 ± 0.3	36.8 ± 0.2	94.5 ± 8.1	238.2 ± 15.4	1.06 ± 0.13
$Irs2^{-/-}$	28.1 ± 2.1	ND	ND	ND	96.1 ± 12.7	294.5 ± 32.0[a]	1.04 ± 0.15
$Irs2^{-/-}p27^{+/-}$	36.4 ± 1.9[b]	ND	ND	ND	85.2 ± 9.0	325.9 ± 21.4	0.81 ± 0.13
$Irs2^{-/-}p27^{-/-}$	39.4 ± 1.7[b]	ND	ND	ND	90.7 ± 15.7	357. 1 ± 23.8	1.19 ± 0.10
$Lepr^{+/-}$	32.4 ± 0.7	1,282.9 ± 75.2[a]	3.8 ± 0.2	36.7 ± 0.3	107.5 ± 7.7	226.5 ± 14.1	1.25 ± 0.13
$Lepr^{-/-}$	62.0 ± 3.6[c]	7,861.9 ± 275.3[c]	7.3 ± 0.7[c]	34.9 ± 0.4[c]	124.0 ± 11.3	368.7 ± 35.2[c]	1.52 ± 0.15
$Lepr^{-/-}p27^{+/-}$	71.9 ± 4.8[d]	8,456.9 ± 292.1	6.7 ± 0.4	35.3 ± 0.4	106.4 ± 10.9	391.3 ± 37.7	1.44 ± 0.19
$Lepr^{-/-}p27^{-/-}$	77.1 ± 5.4[d]	9,978.9 ± 343.3[d]	7.6 ± 0.5	35.1 ± 0.4	81.1 ± 10.9[d]	335.4 ± 31.7	1.31 ± 0.15

Data are means ± s.e.m. from five ($Irs2^{-/-}p27^{-/-}$ or $Lepr^{-/-}p27^{-/-}$) or ten (other genotypes) mice. Total visceral fat mass represents the sum of epididymal, omental and perirenal fat depots. FFA, free fatty acids; ND, not determined. [a]$P < 0.05$ versus wild-type, [b]$P < 0.05$ versus $Irs2^{-/-}$, [c]$P < 0.05$ versus $Lepr^{+/-}$, [d]$P < 0.05$ versus $Lepr^{-/-}$ mice (Student unpaired t-test).

$Irs2^{-/-}$ mice, suggesting the existence of signaling pathways other than that mediated by Akt for regulation of the concentration and subcellular localization of p27. Given that hyperglycemia and reactive oxygen species increase the abundance of p27 in cultured mesangial cells[39] and *in vivo*[39–41], the difference between $Lepr^{-/-}$ and $Irs2^{-/-}$ mice in the extent of hyperglycemia or associated oxidative stress at early stages (7 to 12 weeks) in the development of diabetes may contribute to the increased abundance and nuclear accumulation of p27 in the beta cells of $Lepr^{-/-}$ mice. Secondary abnormalities that are associated with leptin deficiency, such as obesity-induced dysregulation of adipokines, also might contribute to this difference between $Lepr^{-/-}$ and $Irs2^{-/-}$ mice.

Deletion of *Cdkn1b* ameliorated the abnormalities in glucose metabolism in $Irs2^{-/-}$ and $Lepr^{-/-}$ mice. Both insulin resistance and beta-cell dysfunction are responsible for the occurrence of diabetes in these mice. Deletion of *Cdkn1b* had no effect on insulin resistance in $Irs2^{-/-}$ mice, as determined by an insulin tolerance test (**Supplementary Fig. 9** online). Immunoblot analysis also failed to detect an increase in p27 abundance in other organs and tissues, including the brain, liver, adipose tissue and muscle, of $Lepr^{-/-}$ mice (**Supplementary Fig. 10** online). Together with the increased beta-cell mass and enhanced compensatory hyperinsulinemia that are induced by *Cdkn1b* deletion in $Irs2^{-/-}$ and $Lepr^{-/-}$ mice, these data indicate that pancreatic beta cells may be the major site of p27 action during progression of diabetes in $Irs2^{-/-}$ and $Lepr^{-/-}$ mice. We did find, however, that the number of smaller adipocytes was increased by *Cdkn1b* deletion in $Lepr^{-/-}$ mice (data not shown), a change that might possibly ameliorate insulin resistance.

In human diabetes, inadequate beta-cell proliferation in the presence of increased insulin resistance contributes to the pathogenesis of beta-cell failure[1–4]. Postmortem immunostaining of human pancreases with antibodies to p27 has shown marked accumulation of p27 in the nuclei of islet cells in some individuals with type 2 diabetes (T.U., J. Miyagawa, Y.H. and M.K., unpublished data). Although the qualitative nature of these observations requires caution in their interpretation, they are consistent with the possibility that a disturbance in the expression and subcellular localization of p27 may be involved in the development of beta-cell failure in human type 2 diabetes mellitus.

Here we show that increased accumulation of p27, an inhibitor of cell cycle progression through G1, in the nucleus of beta cells contributes to the pathogenesis of type 2 diabetes mellitus in two mouse models. Deletion of *Cdkn1b* ameliorated hyperglycemia by inducing the proliferation of beta cells in the diabetic mice. Our findings thus indicate that cell cycle regulatory reagents, such as p27 inhibitors, might provide new tools for the treatment of type 2 diabetes.

METHODS

Mice. A pBlueScript vector containing the promoter of the rat insulin gene was kindly provided by P.L. Herrera (University of Geneva Medical School, Geneva, Switzerland)[42]. We isolated a human *Cdkn1b* cDNA from HEK293 cells by the polymerase chain reaction (PCR) and inserted it into the *Cla*I and *Eco*RI sites of the vector downstream of the insulin gene promoter. We linearized the construct and microinjected it into pronuclear oocytes of C57BL/6J mice in the Laboratory for Animal Resources and Genetic Engineering (Riken, CDB). We screened the resulting offspring for transgene transmission by PCR and Southern hybridization.

Generation and genotyping of *Cdkn1b* and *Irs2* knockout mice have been described[8,15,43]. We maintained $Irs2^{-/-}$ mice on a mixed C57BL/6J × 129Sv background. We obtained $Lepr^{+/-}$ mice on the C57BL/KsJ background from Clea Japan and crossed them with $Cdkn1b^{+/-}$ mice on the C57BL/6J background to obtain $Lepr^{+/-}Cdkn1b^{+/-}$ mice on a mixed C57BL/6J × C57BL/KsJ background. We intercrossed the double-heterozygote F_1 offspring ($Irs2^{+/-}Cdkn1b^{+/-}$ and $Lepr^{+/-}Cdkn1b^{+/-}$ mice). The double-knockout progeny were observed at the expected Mendelian frequency in both instances.

We maintained the mice as described[44]. For determination of food intake, we housed 9-week-old mice individually and measured the weight of chow remaining in the wire-mesh feeding jar after 5 d. We measured rectal temperature at the end of this 5-d period. We determined blood glucose as well as serum insulin, triglyceride and free fatty acid concentrations as described[44]. We determined the serum concentration of total cholesterol with an Amplex Red Cholesterol Assay kit (Molecular Probes). After killing the mice, we removed and weighed several organs. Unless indicated, we carried out all experiments with male mice. This project was approved by the Animal Ethics Committee of Kobe University Graduate School of Medicine.

Histology, immunostaining and immunoblot analysis. We placed the pancreas in Bouin solution, embedded it in paraffin and sectioned it at a thickness of 4–5 μm. We stained the sections with antibodies to p27 (Transduction Laboratories), Pcna, insulin, glucagon, somatostatin, pancreatic polypeptide or human p27 (all from Dako). We visualized the immune complexes with the use of EnVision/HRP or EnVision/AP kits (Dako). For double staining, we used diaminobenzidine tetrahydrochloride (brown) and fuchsin (red) as the substrates for horseradish peroxidase and alkaline phosphatase, respectively. Alternatively, we visualized immune complexes with Cy3- or fluorescein isothiocyanate–conjugated secondary antibodies (Jackson Immunoresearch) and confocal microscopy. We determined islet mass [(total islet area/pancreas area) × pancreas weight] and islet density (total islet number/pancreas area) with Scion Image software (Scion). We determined the area of individual beta cells with LSM Image Browser software (Carl Zeiss). Pictures shown here are representative of at least 50 islets from indicated genotypes.

For immunoblot analysis, we prepared lysates of isolated islets or Min6 cells as described[45]. We dissolved insoluble material in 25 mM Tris-HCl (pH 7.4) and 1% SDS. We subjected the extracts (5 μg of protein) or Irs2 immunoprecipitates to immunoblot analysis with antibodies to p27 (Transduction Laboratories), $p21^{Cip1}$ (Santa Cruz Biotechnology), Irs2 (Upstate Biotechnology), p85 (Upstate Biotechnology), cleaved caspase-3^{Asp175} (Cell Signaling), β-actin (Sigma-Aldrich) or p27 phosphorylated on Ser^{10} (Zymed Laboratories). We analyzed the immunoblot band intensities with ImageJ software (US National Institutes of Health) and used the actin band to correct for loading differences.

Assay for TUNEL, DNA fragmentation and Akt activity. We carried out TUNEL assays as described[4]. Treatment of pancreatic sections with DNase rendered all nuclei positive in this assay, and such sections were used as positive controls. We carried out DNA fragmentation assays as described[45]; the relative intensity of DNA bands was determined with ImageJ software. We measured the activity of Akt with an assay kit that used glycogen synthase kinase 3α/β (GSK3α/β) as the substrate (Cell Signaling).

Primary culture of beta cells and IGF-1- and leptin-induced p27 translocation. We isolated islets from 8-week-old mice as described[15] and exposed them to trypsin. The dissociated cells were cultured overnight in RPMI 1640 medium supplemented with 10% fetal bovine serum. After deprivation of serum for 4 h, we incubated the cells for 2 h in the absence or presence of recombinant human IGF-1 (30 nM; PeproTech) or recombinant human leptin (30 nM; MP Biomedicals). Then we fixed the cells in 4% paraformaldehyde overnight before staining with antibodies to p27 or to insulin followed by secondary antibodies conjugated with Cy3 or fluorescein isothiocyanate, respectively.

Quantitative PCR coupled with reverse transcription (RT-PCR). We carried out RT-PCR and real-time PCR for analysis of p27 mRNA with total RNA isolated from three mice of each genotype as described[44].

Statistical analysis. Quantitative data are presented as the mean ± s.e.m. from at least three independent experiments, five mice or 200 islets, unless indicated. We assessed interactions among variables by two-way analysis of variance and used the Student's *t*-test to compare independent means. A P value of <0.05 was considered statistically significant.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Deng, S. *et al.* Structural and functional abnormalities in the islets isolated from type 2 diabetic subjects. *Diabetes* **53**, 624–632 (2004).
2. Sakuraba, H. *et al.* Reduced β-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia* **45**, 85–96 (2002).
3. Clark, A. *et al.* Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes. *Diabetes Res.* **9**, 151–159 (1988).
4. Butler, A.E. *et al.* β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes* **52**, 102–110 (2003).
5. Kulkarni, R.N. *et al.* Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* **96**, 329–339 (1999).
6. Kulkarni, R.N. *et al.* β-cell-specific deletion of the Igf1 receptor leads to hyperinsulinemia and glucose intolerance but does not alter β-cell mass. *Nat. Genet.* **31**, 111–115 (2002).
7. Xuan, S. *et al.* Defective insulin secretion in pancreatic β cells lacking type 1 IGF receptor. *J. Clin. Invest.* **110**, 1011–1019 (2002).
8. Withers, D.J. *et al.* Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* **391**, 900–904 (1998).
9. Kubota, N. *et al.* Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory β-cell hyperplasia. *Diabetes* **49**, 1880–1889 (2000).
10. Tuttle, R.L. *et al.* Regulation of pancreatic β-cell growth and survival by the serine/threonine protein kinase Akt1/PKB α. *Nat. Med.* **7**, 1133–1137 (2001).
11. Bernal Mizrachi, E., Wen, W., Stahlhut, S., Welling, C.M. & Permutt, M.A. Islet β cell expression of constitutively active Akt1/PKB α induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J. Clin. Invest.* **108**, 1631–1638 (2001).
12. Garofalo, R.S. *et al.* Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB β. *J. Clin. Invest.* **112**, 197–208 (2003).
13. Brunet, A. *et al.* Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857–868 (1999).
14. Nakae, J. *et al.* Regulation of insulin action and pancreatic β-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat. Genet.* **32**, 245–253 (2002).
15. Kitamura, T. *et al.* The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic β cell growth. *J. Clin. Invest.* **110**, 1839–1847 (2002).
16. Sherr, C.J. & Roberts, J.M. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501–1512 (1999).
17. Rane, S.G. *et al.* Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-islet cell hyperplasia. *Nat. Genet.* **22**, 44–52 (1999).
18. Nakayama, K. *et al.* Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**, 707–720 (1996).
19. Medema, R.H., Kops, G.J., Bos, J.L. & Burgering, B.M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782–787 (2000).
20. Viglietto, G. *et al.* Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nat. Med.* **8**, 1136–1144 (2002).
21. Shin, I. *et al.* PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat. Med.* **8**, 1145–1152 (2002).
22. Liang, J. *et al.* PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat. Med.* **8**, 1153–1160 (2002).
23. Fujita, N., Sato, S., Katayama, K. & Tsuruo, T. Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. *J. Biol. Chem.* **277**, 28706–28713 (2002).
24. Lee, G.H. *et al.* Abnormal splicing of the leptin receptor in diabetic mice. *Nature* **379**, 632–635 (1996).
25. Chen, H. *et al.* Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* **84**, 491–495 (1996).
26. Clement, K. *et al.* A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* **392**, 398–401 (1998).
27. Hellerstrom, C., Andersson, A. & Gunnarsson, R. Regeneration of islet cells. *Acta Endocrinol. Suppl. (Copenh.)* **205**, 145–160 (1976).
28. Withers, D.J. *et al.* Irs-2 coordinates Igf-1 receptor-mediated β-cell development and peripheral insulin signalling. *Nat. Genet.* **23**, 32–40 (1999).
29. Tanabe, K., Okuya, S., Tanizawa, Y., Matsutani, A. & Oka, Y. Leptin induces proliferation of pancreatic β cell line MIN6 through activation of mitogen-activated protein kinase. *Biochem. Biophys. Res. Commun.* **241**,765–768 (1997).
30. Zhou, B.P. *et al.* Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat. Cell Biol.* **3**, 245–252 (2001).
31. Pagano, M. *et al.* Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* **269**, 682–685 (1995).
32. Cunningham, M.A., Zhu, Q. & Hammond, J.M. Foxo1a can alter cell cycle progression by regulating the nuclear localization of p27kip in granulosa cells. *Mol. Endocrinol.* **18**, 1756–1767 (2004).
33. Boehm, M. *et al.* A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression. *EMBO J.* **21**, 3390–3401 (2002).
34. Ishida, N. *et al.* Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. *J. Biol. Chem.* **277**, 14355–14358 (2002).
35. Sheaff, R.J., Groudine, M., Gordon, M., Roberts, J.M. & Clurman, B.E. Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev.* **11**, 1464–1478 (1997).
36. Vlach, J., Hennecke, S. & Amati, B. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. *EMBO J.* **16**, 5334–5344 (1997).
37. Montagnoli, A. *et al.* Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev.* **13**, 1181–1189 (1999).
38. Fujita, N., Sato, S. & Tsuruo, T. Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinase promotes its binding to 14-3-3 and cytoplasmic localization. *J. Biol. Chem.* **278**, 49254–49260 (2003).
39. Wolf, G. *et al.* Glomerular expression of p27Kip1 in diabetic db/db mouse: role of hyperglycemia. *Kidney Int.* **53**, 869–879 (1998).
40. Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813–820 (2001).
41. Wolf, G., Wenzel, U., Hannken, T. & Stahl, R.A.K. Angiotensin II induces $p27^{kip1}$ expression in renal tubules *in vivo*: role of reactive oxygen species. *J. Mol. Med.* **79**, 382–389 (2001).
42. Herrera, P.L. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* **127**, 2317–2322 (2000).
43. Schreyer, S.A., Chua, S.C. Jr. & LeBoeuf, R.C. Obesity and diabetes in TNF-α receptor-deficient mice. *J. Clin. Invest.* **102**, 402–411 (1998).
44. Matsumoto, M. *et al.* PKCλ in liver mediates insulin-induced SREBP-1c expression and determines both hepatic lipid content and overall insulin sensitivity. *J. Clin. Invest.* **112**, 935–944 (2003).
45. Uchida, T., Myers, M.G. Jr. & White, M.F. IRS-4 mediates PKB signaling during insulin stimulation without promoting anti-apoptosis. *Mol. Cell. Biol.* **20**, 126–138 (2000).