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Article

Oxidative Stress Contributes to Aging by Enhancing Pancreatic Angiogenesis and Insulin Signaling

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SUMMARY

JunD, a transcription factor of the AP-1 family, protects cells against oxidative stress. Here, we show that *junD*$^{-/-}$ mice exhibit features of premature aging and shortened life span. They also display persistent hypoglycemia due to enhanced insulin secretion. Consequently, the insulin/IGF-1 signaling pathways are constitutively stimulated, leading to inactivation of FoxO1, a positive regulator of longevity. Hyperinsulinemia most likely results from enhanced pancreatic islet vascularization owing to chronic oxidative stress. Indeed, accumulation of free radicals in β cells enhances *VEGF-A* transcription, which in turn increases pancreatic angiogenesis and insulin secretion. Accordingly, long-term treatment with an antioxidant rescues the phenotype of *junD*$^{-/-}$ mice. Indeed, dietary antioxidant supplementation was protective against pancreatic angiogenesis, hyperinsulinemia, and subsequent activation of insulin signaling cascades in peripheral tissues. Taken together, these data establish a pivotal role for oxidative stress in systemic regulation of insulin and define a key role for the JunD protein in longevity.

INTRODUCTION

The accumulation of reactive oxygen species (ROS) beyond the homeostatic threshold is linked to the appearance of age-related diseases, including cancer (for review, see Balaban et al., 2005; Frisard and Ravussin, 2006). Nearly 50 years ago, Harman proposed the "free radical theory of aging," which suggests that aging and associated degenerative diseases result from the deleterious effects of free radicals on numerous cellular components including lipids, proteins, and nucleic acids (Harman, 1956). Today, although aerobic metabolism and the corresponding generation of ROS remain the most widely accepted cause of aging, substantial unknowns persist. Interestingly, the insulin/IGF-1 pathways, which have been defined as key regulators of longevity in various species, also modulate resistance against ROS (reviewed in Kenyon, 2005; Warner, 2005; Murakami, 2006; Giannakou and Partridge, 2007). Binding of insulin/insulin-like signaling molecules to their tyrosine kinase receptors triggers the recruitment and subsequent activation of phosphatidylinositol 3-kinase (PI3K), which in turn activates several serine/threonine kinases including Akt (for review, see Manning and Cantley, 2007). The FoxO family of transcription factors is a direct substrate of Akt in response to insulin (for reviews, see Arden, 2007; Carter and Brunet, 2007; Huang and Tindall, 2007). Akt phosphorylates conserved residues in FoxO proteins, triggers their export to the cytoplasm, and inactivates them. In *C. elegans*, the extended life span of insulin-like signaling mutants is lost when the worm FoxO ortholog DAF-16 is inactivated (Lin et al., 1997; Ogg et al., 1997). FoxO or DAF-16 induces a genetic program that promotes both resistance to oxidative stress and longevity (for reviews, see Baumeister et al., 2006; van der Horst and Burgering, 2007). Taken together, these data elucidate how insulin/IGF-1 signaling modulates ROS levels and life span through the conserved PI3K/Akt/FoxO signal transduction pathway.

The AP-1 transcription factor plays a key role in regulating cell growth and environmental stress responses. AP-1 is composed of homo- or heterodimers between Jun (c-Jun, JunB, and JunD) and Fos or ATF family members (for review, see Mechta-Grigoriou et al., 2001). Recently, we discovered a new function of JunD in controlling oxidative stress and the angiogenic switch (Gerald et al., 2004; Pouyssegur and Mechta-Grigoriou, 2006). JunD protects cells against oxidative stress by regulating genes involved in antioxidant defense and H_2O_2 production. Using *junD*-deficient fibroblasts, we uncovered a molecular mechanism linking oxidative stress to angiogenesis. Accumulation of H_2O_2 reduces the activity of HIF-prolyl hydroxylases (PHDs), which in turn signal hypoxia-inducible factor (HIF) α subunits for degradation. Subsequently, HIF-α proteins accumulate and enhance the transcription of *VEGF-A* (vascular endothelial growth factor A), a potent proangiogenic factor. Thus, our previous study determined that JunD protects cells from oxidative stress and exerts an antiangiogenic effect.

Angiogenesis has recently been shown to play an essential part in modulating insulin production. Crosstalk between endothelial and endocrine cells is crucial for endocrine gland function since endocrine cells sense molecules in the bloodstream and

secrete others in response. Pancreatic β cells promote local vascular development by secreting VEGF-A (Inoue et al., 2002; Lammert et al., 2003b; Brissova et al., 2006). Endothelial cells signal back to endocrine cells by producing extracellular matrix (ECM) and promoting β cell function (Duvillie et al., 2006; Nikolova et al., 2006). Mice that are genetically depleted of islet capillaries exhibit lower levels of insulin expression and secretion (Brissova et al., 2006; Nikolova et al., 2006). These observations support previous studies showing that a proper vasculature is necessary for the initial induction of insulin synthesis during embryogenesis (Lammert et al., 2001, 2003a; Yoshitomi and Zaret, 2004).

Consistent with the notion that resistance to oxidative stress is highly coupled to life-span extension, we show here that inactivation of *junD* decreases longevity in mice. This effect is mediated at least in part by an increase in pancreatic angiogenesis and subsequent enhanced insulin production. Stimulation of the insulin-dependent signaling pathways inactivates FoxO1, which conceivably accelerates aging. Interestingly, the phenotype that results from constitutive oxidative stress due to *junD* deletion is rescued to a large extent by long-term antioxidant treatment. Our study thus identifies an angiogenic mechanism by which oxidative stress can modulate insulin/IGF-1 signaling and, in turn, life span.

RESULTS

Inactivation of *junD* Decreases Life Span in Mice

We recently demonstrated that JunD protects cells against oxidative stress, thereby reducing tumor angiogenesis (Gerald et al., 2004). To further investigate the role of JunD in normal physiology, we monitored a cohort of *junD*-deficient and wild-type (WT) mice over their entire life span. At birth, $junD^{-/-}$ mice were indistinguishable from their WT littermates. However, at 2 months of age, they exhibited growth retardation, as evidenced by reduced body weight. Indeed, while the weight of WT animals increased constantly, the weight of mutants stopped increasing (Figure 1A). In contrast, the body length of *junD*-deficient animals remained normal and comparable to controls (see Figure S1A available online). Furthermore, when fed ad libitum on a standard diet (5% fat) and maintained in regular housing until natural death, the mutant mice exhibited a decrease in mean (17%) and maximum (14%) life span relative to WT littermates ($p = 0.0005$) (Figure 1B). No obvious difference was observed between heterozygous and WT mice (data not shown). Long-term follow-up of *junD*-deficient mice revealed a striking premature-aging phenotype. *junD*-deficient mice had a normal appearance until the age of ~6 months, when we first noted cataracts, slight hair loss, and graying. As $junD^{-/-}$ animals aged, mono- or bilateral cataracts were frequently observed, and varying degrees of cachexia, alopecia (loss of body hair), and lordokyphosis (abnormal curvature of the spine) became evident (Figure 1C). Finally, postmortem examination revealed that the prematurely aging $junD^{-/-}$ mice developed various types of neoplasia, with the most frequent cause of death being high-grade diffuse lymphomas (average incidence 23%) (Figure 1D). Since lymphomas were also detected at a similar frequency in old WT mice (data not shown), they were considered as a feature of premature aging. These results indicate that *junD* inactivation in mice is associated with reduced life span and premature onset of aging-related syndromes.

$junD^{-/-}$ Mice Suffer from Persistent Hyperinsulinemia

Since metabolism may play an important role in controlling aging, we explored several blood parameters in $junD^{-/-}$ mice and compared them to WT animals. We repeatedly observed that mice defective in *junD* expression had reduced blood glucose (Figure 2A). In the fed state, $junD^{-/-}$ animals had 23% lower blood glucose levels than control animals ($p < 0.005$) (Figure 2A, left panel). Similarly, in the fasted state, even though blood glucose levels declined in both control and mutant mice, glucose values remained 20% lower in *junD*-deficient animals compared to WT littermates ($p < 0.01$) (Figure 2A, right panel). $junD^{-/-}$ mice suffered from chronic hypoglycemia immediately after the weaning age, and no sex-dependent differences were observed (Figure 2B). Ability to handle a glucose load was assessed by carrying out an intraperitoneal glucose tolerance test (i.p.GTT) at 6 months of age (Figure 2C). Although blood glucose levels remained lower in $junD^{-/-}$ mice compared to control animals, no significant difference was observed in the i.p.GTT response between WT and *junD*-deficient mice, indicating that the $junD^{-/-}$ mice did not suffer from glucose intolerance or insulin resistance. We next measured food and water intake but did not detect significant differences between the two genotypes (Figure S1B). Since hypoglycemia and reduced body mass despite normal food intake are phenotypes shared by long-lived mice with reduced secretion of IGF-1 (Bartke and Brown-Borg, 2004), we next investigated the IGF-1 levels. Serum IGF-1 levels were comparable in $junD^{-/-}$ and WT mice (Figure 2D). Moreover, normal body length in *junD*-deficient mice excluded a potential role for pituitary or growth hormone receptor function.

As the ratio of insulin to glucagon is critical in maintaining euglycemia, we next determined the serum levels of these hormones in WT and $junD^{-/-}$ mice (Figure 2E). Interestingly, blood insulin levels in the mutant mice were higher than in controls. In contrast, glucagon levels remained comparable between the two genotypes. Consequently, the ratio of insulin to glucagon was inappropriately high considering the mutants' severe hypoglycemia (Figure 2E). To further explore the link between JunD and metabolic abnormalities, we analyzed its expression pattern in pancreas (Figure 2F). Interestingly, *junD* expression was restricted to clustering islet cells and was not detected in the exocrine tissue. Virtually all insulin-secreting β cells robustly expressed *junD*, while glucagon-positive α cells did not (Figure 2F). Moreover, in accordance with plasma levels, the steady-state insulin content was higher in $junD^{-/-}$ islets than in WT pancreas. Furthermore, the pancreatic glucagon content did not change significantly between the two genotypes (Figure 2G). Hence, enhanced pancreatic and plasma insulin levels in $junD^{-/-}$ mice account for their chronic hypoglycemia. Moreover, the pattern of *junD* expression in the pancreas is consistent with a direct effect on insulin production.

Increased Angiogenesis in $junD^{-/-}$ Pancreatic Islets

Insulin secretion from pancreatic β cells is a tightly regulated process that couples the uptake of glucose with ATP-sensitive depolarization of the cell membrane to stimulate exocytosis of insulin-containing granules (Yang and Berggren, 2006). We measured ATP levels but did not observe any increase in ATP levels in $junD^{-/-}$ mice that could account for chronic release of insulin (Figure S2A). Moreover, steady-state mRNA levels of

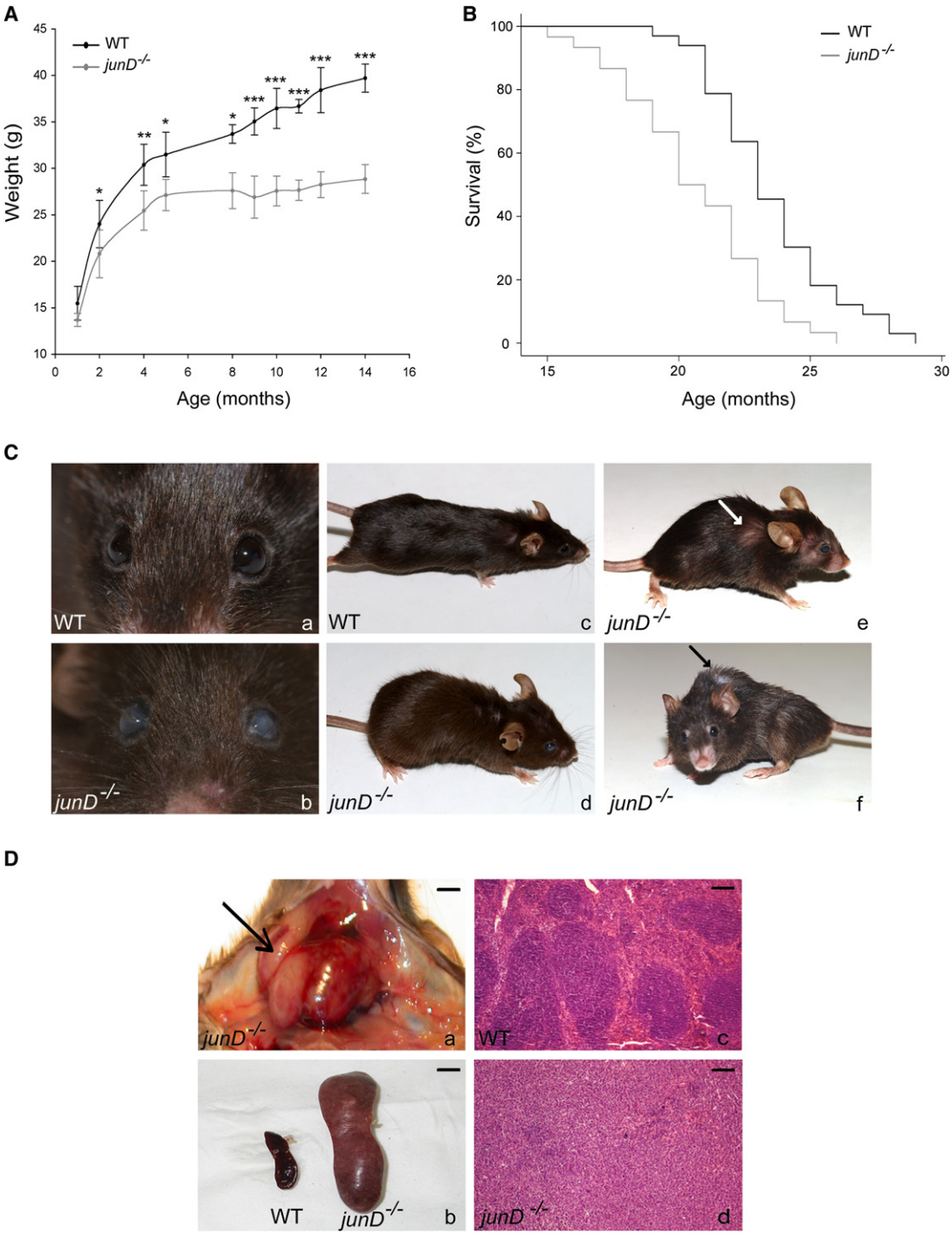

Figure 1. Senescence Features and Early Death of *junD*$^{-/-}$ Mice

(A) Age-dependent changes in body weight of *junD*-deficient mice (*junD*$^{-/-}$; gray line) and wild-type animals (WT; black line) (n = 10).

(B) Kaplan-Meyer survival curve of *junD*$^{-/-}$ mice (gray line) and WT littermate controls (black line) (n = 35) (p = 0.0005, log-rank test).

(C) General appearance of 12-month-old WT and *junD*$^{-/-}$ mice. *junD*$^{-/-}$ mice suffer from cataracts (Cb), cachexia (Cd), alopecia (Ce, arrow), and lordokyphosis (Cf, arrow) compared to WT animals (Ca and Cc).

(D) High-grade diffuse lymphomas in *junD*$^{-/-}$ mice are characterized by lymphadenopathy (Da), splenomegaly (Db), and diffuse organization of the spleen (Dd), with no visible follicles as detected in WT animals (Dc).

n represents the number of animals/condition/genotype. All values are presented as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.005 by Student's t test.

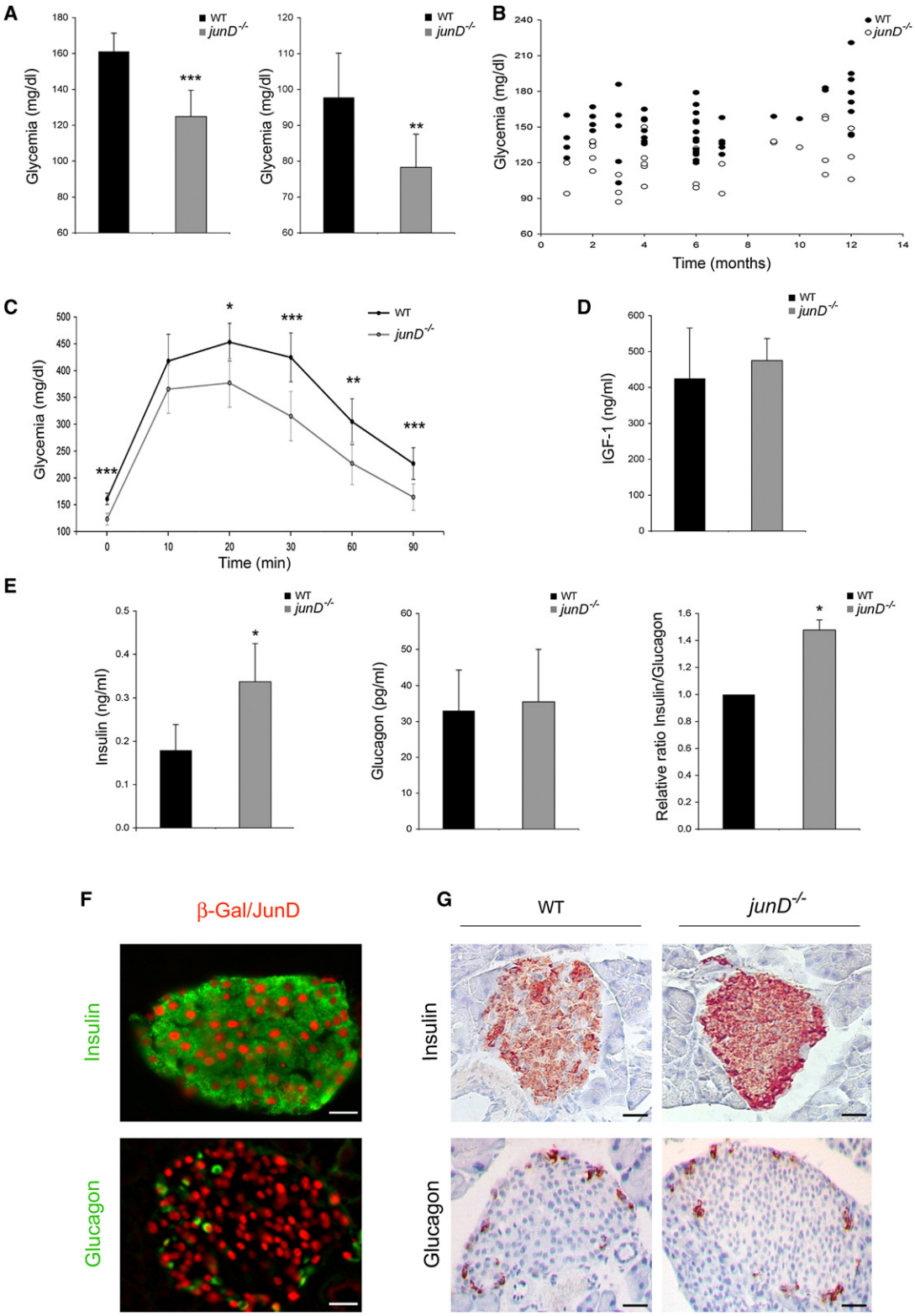

Figure 2. Metabolic Defects in *junD*-Deficient Mice

(A) Blood glucose levels in fed (left panel) and fasted (right panel) WT and $junD^{-/-}$ mice (n = 12).

(B) Variation of blood glucose levels with age in $junD^{-/-}$ mice (○) relative to WT counterparts (•).

(C) Glucose tolerance tests after intraperitoneal administration of glucose load in 6-month-old animals of the indicated genotype (n = 9).

(D) Levels of plasma IGF-1 in fasted WT and $junD^{-/-}$ mice (n = 7).

glucose-sensing insulin secretion partners (including glucose transporter 2 [*Slc2a2/Glut2*], glucokinase [*Gck*], glutamate dehydrogenase [*Glud1*], and both subunits of the ATP-dependent K^+ channel [*Kcnj11/Kir6.1* and *Abcc8/SUR1*]) revealed no significant changes (Figure S2B), suggesting that JunD regulates a distinct process in β cells that contributes to the phenotype.

Interestingly, although *junD*-deficient mice displayed a macroscopically normal pancreas (Figure S2C), *junD*$^{-/-}$ islets exhibited an increase in vascularization as compared to those of WT animals (Figure 3A). Histological analysis of sections stained with endothelial- and smooth muscle cell-specific antibodies showed that the islets of *junD*$^{-/-}$ mutants exhibited increased staining in both cell types compared to controls (Figure 3A). By monitoring the vascular density, we observed that the vascular content was significantly increased in *junD*-deficient islets relative to controls (Figure 3B). Since we recently demonstrated that *junD* inactivation enhances the expression of *VEGF-A*, a major proangiogenic factor, by stabilizing its master regulator HIF-1α (Gerald et al., 2004), we explored whether HIF-1α accumulates in *junD*$^{-/-}$ β cells and stimulates the expression of *VEGF-A*. Immunohistochemistry using HIF-1α antibody revealed that HIF-1α protein levels were higher in *junD*$^{-/-}$ β cells relative to controls (Figure 3C). Furthermore, expression of *VEGF-A* was strongly increased in *junD*-deficient β islets (Figure 3C). Consistent with previous studies (Duvillie et al., 2006; Nikolova et al., 2006), we found that an increase in the number of vascular cells in *junD*$^{-/-}$ pancreas resulted in the formation of capillaries enriched in basement membrane proteins, such as fibronectin, next to the β cells (Figure 3C).

Recent studies have shown that endothelial cell signals promote insulin expression and secretion in adult islets (Brissova et al., 2006; Nikolova et al., 2006). Consistently, both insulin 1 (*Ins1*) and insulin 2 (*Ins2*) mRNA levels were increased in *junD*$^{-/-}$ pancreas compared to WT (Figure 3D). Moreover, since there is a positive relationship between cytosolic Ca^{2+} and insulin release (reviewed in Yang and Berggren, 2006), we examined changes in cytosolic free Ca^{2+} levels in response to glucose from isolated WT and *junD*$^{-/-}$ islets. Under basal conditions (2.8 mM glucose), resting Ca^{2+} concentration was significantly higher in *junD*-deficient islets compared to controls ($p < 0.005$) (Figure 3E), suggesting an enhanced constitutive secretory pathway, which could contribute to basal hyperinsulinemia. In addition, free Ca^{2+} levels reached higher levels after glucose stimulation (16 mM) in *junD*-deficient islets relative to WT cells. Taken together, these data show that *junD*-deficient pancreas accumulates the HIF-1α transcription factor, inducing *VEGF-A* expression and β islet microvasculature. Consequently, the angiogenic switch induced in *junD*$^{-/-}$ pancreas may play a critical role in insulin synthesis and Ca^{2+}-dependent insulin secretion.

Increased Angiogenesis in *junD*$^{-/-}$ Pancreas Results from Chronic Oxidative Stress

We and others have shown that the stability of the proangiogenic factor HIF-1α is sensitive to redox status in well-oxygenated cells (for review, see Pouyssegur and Mechta-Grigoriou, 2006). Since we previously established that JunD regulates genes involved in antioxidant defense (Gerald et al., 2004), we evaluated ROS content in WT and *junD*$^{-/-}$ pancreas using a cell-permeable molecule that fluoresces when oxidized by peroxides (CM-H_2DCFDA) (Figure 4A). Interestingly, the ROS-specific signal exhibited a 2-fold increase in *junD*$^{-/-}$ pancreas compared to WT. To further assess the role of constitutive oxidative stress in persistent hypoglycemia, we subjected WT and *junD*$^{-/-}$ mice to long-term antioxidant treatment. N-acetylcysteine (NAC) was chosen because it is well tolerated in animals and can be orally administered over long periods of time. We first confirmed that long-term administration of NAC decreased ROS content in *junD*$^{-/-}$ pancreas (Figure 4B). In addition, NAC completely restored blood glucose and plasma insulin content to normal levels in *junD*-deficient mice (Figures 4C and 4D) and had no effect on glucagon levels (Figure 4E). Finally, long-term NAC treatment significantly reduced pancreatic angiogenesis in *junD*-deficient animals to WT levels (Figures 4F and 4G). Thus, dietary supplementation with antioxidant treatment turned out to be protective against pancreatic angiogenesis, hyperinsulinemia, and hypoglycemia in *junD*$^{-/-}$ mice. In addition, NAC treatment restored the body weight of *junD*$^{-/-}$ mice to normal (Figure 4H). Moreover, 10-month-old NAC-treated *junD*$^{-/-}$ mice did not exhibit any signs of premature aging, including cataracts, hair loss, or graying (data not shown). This shows that antioxidant treatment also rescued the premature-aging phenotype of *junD*$^{-/-}$ mice, although definitive proof concerning premature death may require an additional treatment period of 15–20 months. In conclusion, these data suggest that enhanced pancreatic angiogenesis and subsequent increased insulin secretion and premature aging result from chronic oxidative stress in *junD*-deficient mice, a phenotype that is rescued to a large extent by long-term antioxidant treatment.

Activation of Insulin-Dependent Pathways in *junD*$^{-/-}$ Mice

Since insulin signaling pathways are key regulators of longevity, we next investigated to what extent the increased insulin levels in *junD*$^{-/-}$ mice affect intracellular signaling in peripheral insulin-responsive tissues such as liver (Figure 5). We chose the liver because JunD is not expressed in hepatocytes (Thepot et al., 2000 and data not shown), enabling detection of circulating insulin independent of the constitutive oxidative stress caused by *junD* deletion.

Western blot analysis showed a marked increase in tyrosine phosphorylation of the insulin receptor (IR) in the liver of *junD*$^{-/-}$ mice compared to controls (Figure 5A). As expected, insulin-induced phosphorylation of Akt was also markedly increased in *junD*-deficient tissues relative to WT tissues (Figure 5A). Furthermore, since FoxO1 has been identified as a major component of insulin signaling, we investigated the level and degree of its phosphorylation in *junD*$^{-/-}$ liver. Consistently, the levels of FoxO1

(E) Levels of plasma insulin (left panel) and glucagon (middle panel) and ratio of insulin to glucagon (right panel) in fasted WT and *junD*$^{-/-}$ mice (n = 13).
(F) Double immunohistochemistry of pancreas sections from adult mice detecting β-gal/JunD and insulin (upper panel) or glucagon (lower panel).
(G) Representative immunohistochemistry showing increased insulin and equivalent glucagon levels in *junD*$^{-/-}$ pancreatic islets relative to WT.
Scale bars = 20 μm. n represents the number of animals/condition/genotype. All values are presented as means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ by Student's t test.

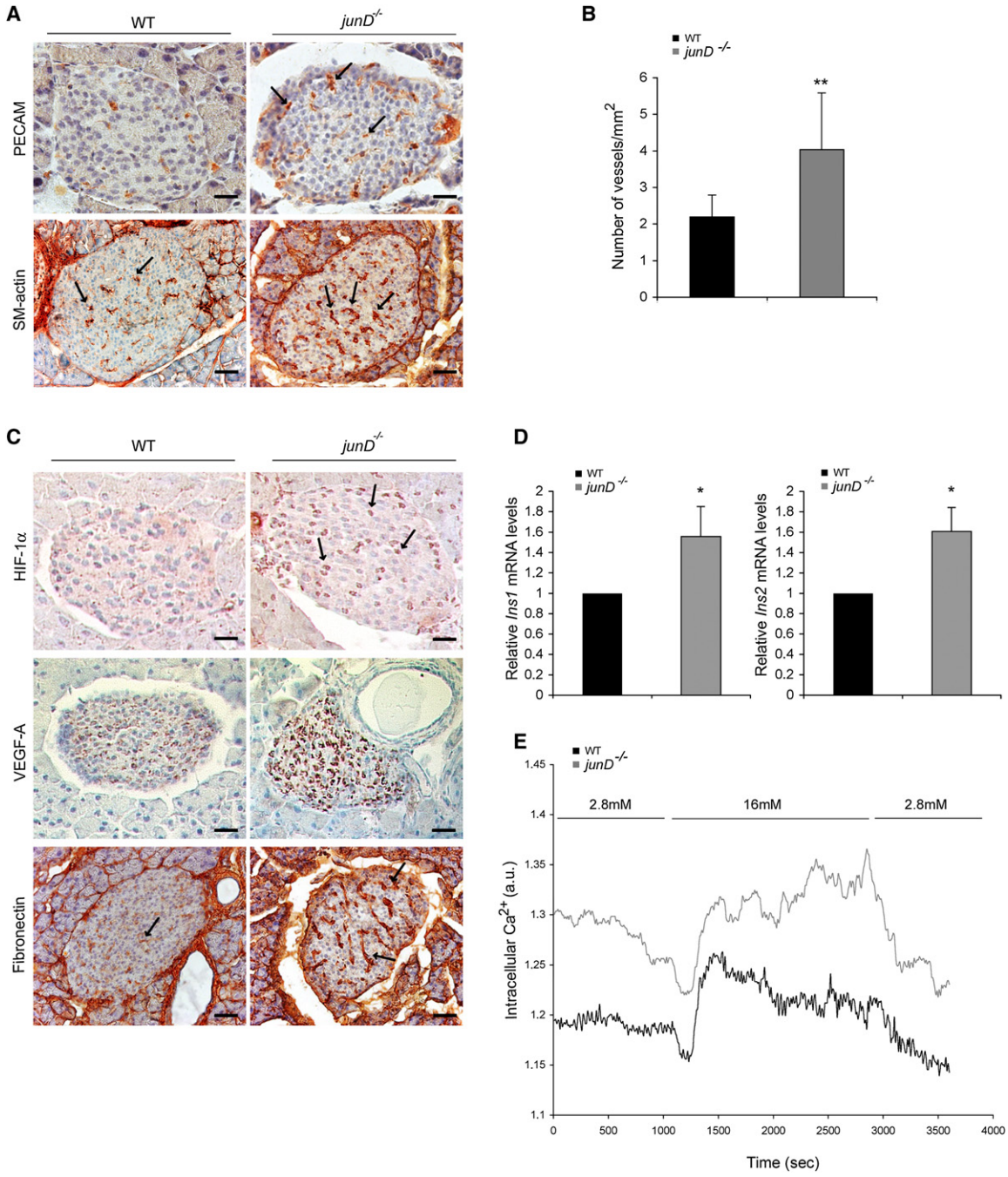

Figure 3. Increased Angiogenesis in *junD*$^{-/-}$ Pancreatic Islets

(A) Representative views of WT and *junD*$^{-/-}$ pancreatic sections stained with specific antibodies as indicated. Arrows indicate typical vessels used for quantification.

(B) Vascular density (vessels/mm^2) in WT and *junD*$^{-/-}$ mice. Data shown are from three independent experiments (n ≥ 10 fields per genotype).

(C) Representative views of WT and *junD*$^{-/-}$ pancreatic sections stained with specific antibodies as indicated.

(D) *Ins1* and *Ins2* mRNA levels as measured by qPCR analysis in WT and *junD*$^{-/-}$ mice (n = 6).

(E) Intracellular calcium measurements in isolated WT and *junD*$^{-/-}$ islets at glucose concentrations of 2.8 mM and 16 mM (n = 3).

Scale bars = 20 μm. n represents the number of animals/condition/genotype. All values are presented as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.005 by Student's t test.

phosphorylation were increased in *junD*-deficient liver while the total amount of FoxO1 protein decreased (Figure 5A), arguing that it is constitutively inactivated and degraded in *junD*-deficient mice. Immunohistochemistry further confirmed that the levels of phospho-FoxO1 were higher in *junD*-deficient hepatocytes compared to controls (Figure 5B). As a consequence, total FoxO1

protein levels were reduced, most probably due to increased degradation resulting from phosphorylation. Finally, we monitored insulin-dependent pathways in NAC-treated mice. Consistent with its effect on blood glucose and insulin levels, NAC reduced the phosphorylation rate of IR, Akt, and FoxO1 to WT levels. Together, these data indicate that overproduction of insulin in *junD*-deficient mice stimulates insulin-dependent cascades in liver, which decreases FoxO1 activity, producing a phenotype that is reversed by antioxidant treatment.

DISCUSSION

Longevity of eukaryotic organisms is modulated, at least in part, by their ability to control metabolism and prevent oxidative damage. In the present study, we have highlighted a key function of persistent oxidative stress in pancreatic β cell function and metabolism using *junD*-deficient mice. Accumulation of ROS in pancreatic β cells increases pancreatic angiogenesis, which presumably stimulates insulin secretion. Consequently, peripheral tissues exhibit constitutive activation of insulin signaling pathways and partial inactivation of the FoxO1 transcription factor, which acts as a mediator of shortened life span. All these features are alleviated by long-term antioxidant treatment, thereby supporting the link between persistent oxidative stress and systemic regulation of insulin (Figure 6).

Endocrine Regulation of Insulin by Oxidative Stress

Although it is now well established that oxidative stress can cause aging through oxidative damages to various cell components, one of the most prevalent systems that has emerged from the search for longevity genes is the insulin/IGF-1 signaling pathways. Since suppression of insulin/IGF-1 signaling extends life span and delays age-dependent functional decline, these hormones have been considered the most likely candidate effectors of aging in diverse organisms. Long-lived mouse mutants with impaired insulin/IGF-1 signaling also exhibit enhanced resistance to oxidative stress (Holzenberger et al., 2003; Kurosu et al., 2005; Yamamoto et al., 2005) (for reviews, see Bartke and Brown-Borg, 2004; Murakami, 2006). In recent years, several studies have substantiated the role of FoxO transcription factors as downstream effectors of the insulin/IGF-1-PI3K-Akt signaling pathway (reviewed in Huang and Tindall, 2007). Upon reduction of insulin/IGF-1-dependent cascades and subsequent inactivation of Akt, FoxO proteins are translocated into the nuclei, leading to upregulation of oxidative-stress resistance genes. Moreover, the p66shc redox enzyme that generates ROS also modulates FoxO activity (Migliaccio et al., 1999; Nemoto and Finkel, 2002). Longevity and resistance to oxidative stress are thus associated with reduced insulin/IGF-1 signaling and enhanced FoxO activity. The fact that *junD*$^{-/-}$ mice suffer from chronic oxidative stress, exhibit persistent hyperinsulinemia and FoxO1 inactivation, and age prematurely is consistent with this hypothesis. These mice constitute an excellent model for identifying new mechanisms linking accumulation of ROS to long-term pathological consequences. Based on *junD*-deficient mice, we propose that ROS and insulin signaling display reciprocal crosstalk. Although insulin/IGF-1 signaling pathways modulate intracellular ROS levels, we suggest here a regulatory feedback loop by which ROS regulate insulin levels. Indeed, we have identified a proangiogenic mechanism by which oxidative stress might control insulin synthesis and insulin-dependent cascades.

Although IGF-1 is clearly an important regulator of life span in mammals (Holzenberger et al., 2003), the relationship between insulin and aging is more complex (for review, see Russell and Kahn, 2007). Some evidence suggests that decreased insulin levels are correlated with longevity. For instance, caloric restriction (CR) in mammals extends life span, and one proposed mechanism by which this occurs is through reduced levels of insulin (reviewed in Masoro, 2005). Indeed, CR cells show resistance to oxidants and increased expression of stress-response genes. Moreover, insulin or IGF-1 supplementation of CR serum reduces resistance to ROS, consistent with the notion that CR and insulin/IGF-1 pathways function in partially overlapping mechanisms. Finally, fat-specific insulin receptor knockout (FIRKO) mice have an increased life span, thereby demonstrating the beneficial effect of reduced insulin signaling in adipose tissue on longevity (Bluher et al., 2003). In addition to demonstrating that insulin signaling can regulate aging, the FIRKO mouse establishes a non-cell-autonomous control of longevity. Consistent with these observations, our data suggest that despite normal IGF-1 levels, increased insulin levels modulate aging. Thus, in addition to oxidative damages, our results suggest a ROS-mediated endocrine regulation of aging.

Increased β Islet Vasculature as a Result of Chronic Oxidative Stress

β cells are ill equipped to dispose of ROS since the level of their antioxidant enzymes is low (Welsh et al., 1995; Lenzen et al., 1996). JunD regulates genes involved in antioxidant defense and H_2O_2 metabolism (Gerald et al., 2004). It thus follows that β cells from *junD*$^{-/-}$ mice show high sensitivity to ROS. Indeed, we detected clear accumulation of ROS in the pancreas of *junD*$^{-/-}$ mice. In contrast, basal ROS levels were not significantly different between the livers of control and mutant mice, in agreement with the fact that *junD* expression is not detected in hepatocytes (G.L. and B.B., unpublished data). We have recently shown that constitutive oxidative stress enhances angiogenesis (Gerald et al., 2004; Pouyssegur and Mechta-Grigoriou, 2006). We show here that ROS accumulation in *junD*$^{-/-}$ β cells increases β islet angiogenesis. Pancreatic microvasculature has been shown to play a pivotal role in endocrine islet development and function (for reviews, see Cleaver and Melton, 2003; Zaret, 2006). Endocrine cells upregulate *VEGF-A* as soon as they start to secrete hormones and signal to the adjacent endothelial cells to promote the formation of a dense network of capillaries. Conversely, capillary endothelial cells within islets produce a basement membrane of ECM that signals back to pancreatic β cells and regulates insulin levels (Duvillie et al., 2006; Nikolova et al., 2006). Accordingly, specific removal of *VEGF-A* in β cells depletes islets of capillaries, and the corresponding mice exhibit lower levels of insulin expression and insulin secretory granules. Moreover, ROS have recently been described as signaling molecules in glucose-stimulated insulin secretion (Pi et al., 2007). We observed a correlation between oxidative stress-mediated ectopic vascularization and increased insulin expression and secretion in *junD*-deficient pancreatic islets, consistent with these aforementioned observations. Intriguingly, although glucose metabolism stimulates glucagon secretion from α cells by a mechanism that mirrors

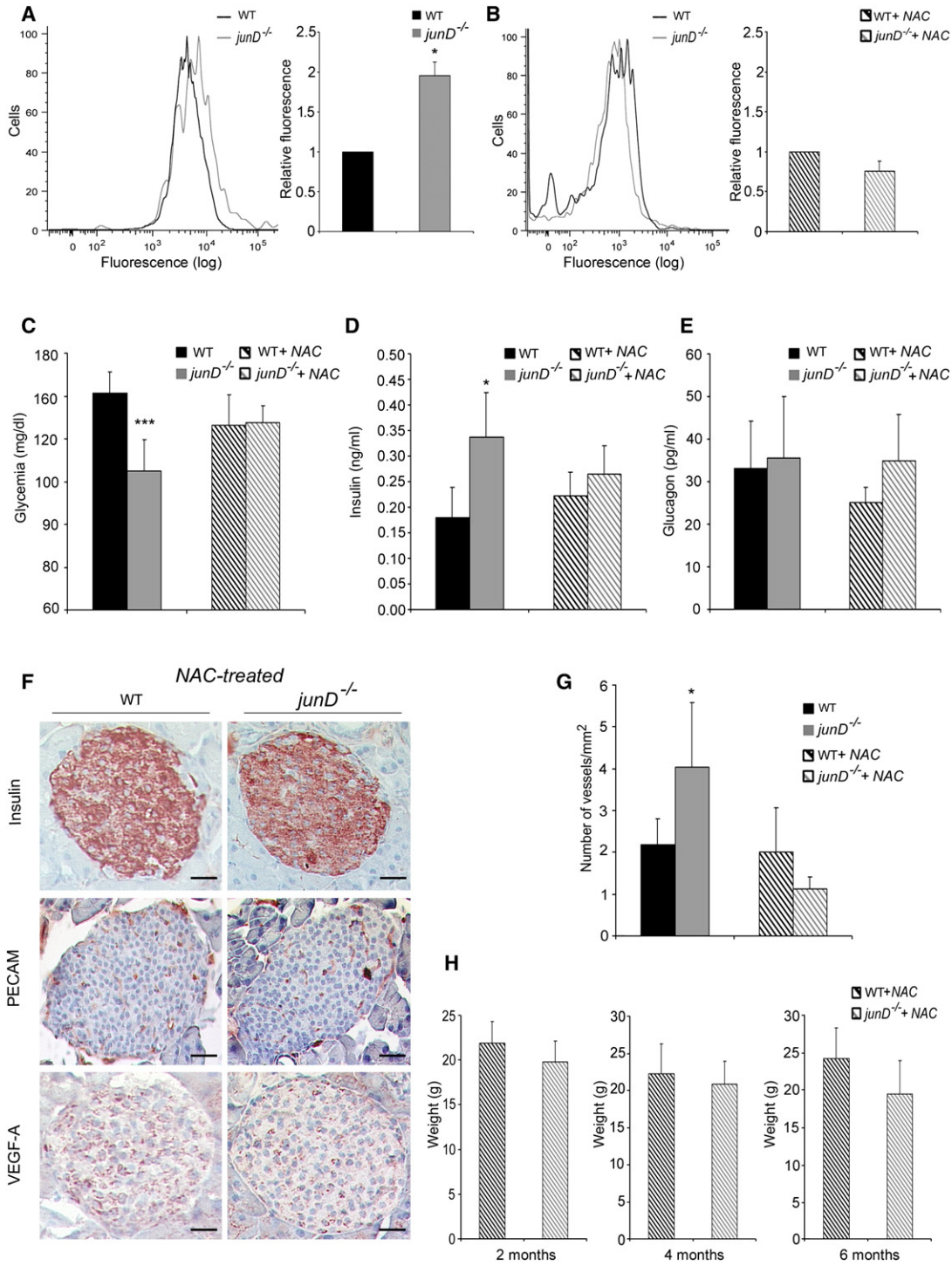

Figure 4. Constitutive Oxidative Stress in *junD*$^{-/-}$ β Islets

(A) Relative reactive oxygen species (ROS) levels in WT and *junD*$^{-/-}$ pancreas as evaluated using the ROS-sensitive fluorescent dye CM-H_2DCFDA (right panel). A representative sample for three independent analyses is displayed (left panel).

(B) Relative ROS levels after long-term N-acetylcysteine (NAC) treatment in WT and *junD*$^{-/-}$ mice (n = 3).

(C) Comparison of blood glucose levels in NAC-treated and untreated WT and *junD*$^{-/-}$ mice (n = 7).

(D) Plasma insulin levels in NAC-treated and untreated WT and *junD*$^{-/-}$ mice (n = 7).

(E) Plasma glucagon levels in NAC-treated and untreated WT and *junD*$^{-/-}$ mice (n = 7).

(F) Representative views of NAC-treated WT and *junD*$^{-/-}$ pancreatic sections stained with specific antibodies as indicated. Scale bars = 20 μm.

(G) Vascular density (vessels/mm^2) in NAC-treated and untreated WT and *junD*$^{-/-}$ mice (n = 3).

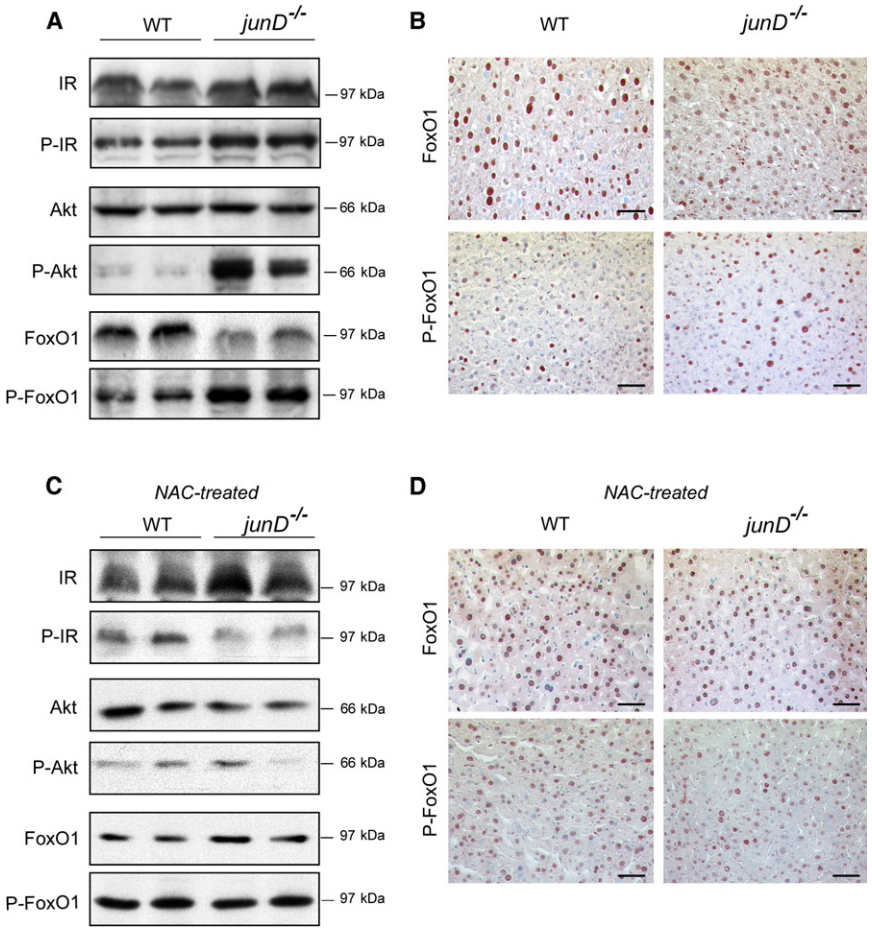

Figure 5. Activation of Insulin Signaling Pathways in Liver of *junD*$^{-/-}$ Mice

(A) Western blot analysis of whole extracts from livers isolated from WT and *junD*$^{-/-}$ mice. Analysis was performed using antibodies specific for the insulin receptor (IR), Akt, FoxO1, and their corresponding phosphorylated isoforms as indicated.

(B) Representative views of liver sections immunostained with specific antibodies recognizing FoxO1 and phospho-FoxO1. Scale bars = 30 μm.

(C) Western blot analysis using antibodies specific for IR, Akt, FoxO1, and their corresponding phosphorylated isoforms in livers isolated from NAC-treated WT and *junD*$^{-/-}$ mice.

(D) Representative views of liver sections from NAC-treated WT and *junD*$^{-/-}$ mice immunostained with FoxO1- and phospho-FoxO1-specific antibodies. Scale bars = 30 μm.

that of β cells (for review, see Gromada et al., 2007), glucagon secretion is not modified in *junD*$^{-/-}$ mice. Since insulin has been shown to inhibit glucagon secretion, we hypothesize that enhanced secretion of insulin in *junD*$^{-/-}$ mice antagonizes the potential prostimulatory effect of angiogenesis on glucagon secretion.

The angiogenic phenotype of *junD*$^{-/-}$ β islets is completely reversed by long-term antioxidant treatment: pancreatic and plasma insulin levels are restored to normal levels after NAC administration. Long-term administration of NAC to *junD*$^{-/-}$ mice confirms the cause-and-effect relationship between oxidative stress, vascularization of pancreatic islets, and insulin secretion and provides a new model for angiogenesis and longevity (Figure 6). Oxidative stress increases islet-secreted *VEGF-A*, which in turn generates a permeable endothelium allowing an increased amount of insulin to pass quickly into the bloodstream. Our data extend previous observations and show that factors that modulate angiogenesis, such as ROS, are likely responsible for the increased amount of insulin delivered into the systemic circulation.

JunD Protein: A Protective Function in Aging and Insulin Signaling

The premature aging and death of *junD*$^{-/-}$ mice are associated with constitutive insulin secretion and signaling. Remarkably, the severity of the symptoms observed in *junD*$^{-/-}$ mice is amplified by the deletion of one copy of the c-*jun* gene (G.L. and F.M.-G., unpublished data), suggesting that the different Jun proteins are master regulators of aging and resistance to oxidative stress in mammals. Since ample genetic data have demonstrated that insulin/IGF-1 pathways modulate life span in various species, it will be of interest to determine whether the function of Jun in controlling life span is conserved in lower organisms, such as *Drosophila* or *C. elegans*.

In this study, we show that JunD modulates insulin-dependent pathways in mice through a ROS-dependent mechanism. Interestingly, ROS have been shown to promote sustained Jun N-terminal kinase (JNK) activation (Kamata et al., 2005). Moreover, JNK controls life span and stress tolerance in *Drosophila* by limiting insulin/IGF-1 signaling (Wang et al., 2003, 2005). In *Drosophila*, JNK has been shown to act at two levels: cell autonomously, by antagonizing insulin/IGF-1 signaling and promoting nuclear localization of Dfoxo, and systemically, by downregulating expression of Dilp2, a peptide that closely resembles insulin (Wang et al., 2005). We show here that this mode of action is also observed with JunD protein in mammals. Indeed, by decreasing insulin levels and insulin signaling in peripheral tissues, JunD influences life span in mice by cell-autonomous and non-cell-autonomous mechanisms.

Pathological Consequences of Pancreatic Oxidative Stress

Neonatal hyperinsulinism is a clinical syndrome of pancreatic β cell dysfunction characterized by failure to suppress insulin secretion despite hypoglycemia (for review, see Glaser, 2000). The disease can be caused by recessive mutations in *Kcnj11/Kir6.1* or *Abcc8/SUR1*, by loss-of-function mutations in *Gck*, or

(H) Body weight of NAC-treated WT and *junD*$^{-/-}$ mice (n = 5).

n represents the number of animals/condition/genotype. All values are presented as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.005 by Student's t test.

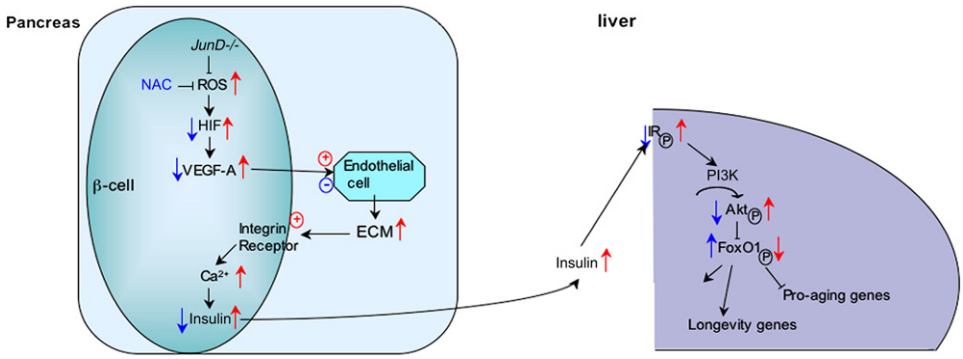

Figure 6. Model for Regulation of Insulin Signaling Pathways by JunD

In mice, JunD regulates the expression of genes involved in the control of ROS levels. JunD is highly expressed in pancreatic β cells that are sensitive to ROS due to low levels of antioxidant defense. Accordingly, *junD* deletion leads to accumulation of H_2O_2 in β cells. As a consequence, ROS promotes an angiogenic switch in β islets by stabilizing HIF-1α and stimulating *VEGF-A*. Endothelial cells secrete large amounts of extracellular matrix (ECM) components that signal back to endocrine cells and increase both insulin expression and Ca^{2+}-dependent exocytosis. The large amount of insulin delivered into the systemic circulation subsequently stimulates insulin signaling pathways in peripheral tissues (including liver) and partially inactivates the FoxO family of transcription factors, a pathway that is known to control life span.

by activating mutations in *Glud1* (Sharma et al., 2000; Straub et al., 2001; Stanley, 2004). However, in 45% of patients, the basis of the pathology has not yet been elucidated. Since we did not detect any variation in the expression of these genes in *junD*$^{-/-}$ mice, our current data suggest that persistent oxidative stress in the pancreas promotes hyperinsulinism and may contribute to the disease.

The metabolic state of hyperinsulinemic patients ranges from recurrent hypoglycemia to insulin-requiring diabetes. Surprisingly, despite prolonged ROS excess, aging *junD*-deficient mice never develop insulin resistance, glucose intolerance, or diabetes. This indicates that although diabetes is typically accompanied by increased production of free radicals, chronic ROS production is not sufficient in and of itself to cause β cell dysfunction and diabetes. Interestingly, insulin resistance in diabetes can be associated with high levels of blood insulin, a mechanism referred to as compensation. Indeed, β cells can attempt to compensate for insulin resistance by increasing insulin secretion, and insufficient compensation leads to the onset of glucose intolerance. Our results suggest that ROS-mediated β islet vascularization may participate in the first phases of this compensatory mechanism and enhance insulin production. ROS-induced pancreatic angiogenesis may be one mechanism used by the organism to try to circumvent hyperglycemia. Future studies should investigate whether increased pancreatic vascularization due to *junD* inactivation may affect the severity of diabetes progression and obesity in models such as *ob/ob* mice. If this hypothesis is further confirmed, stimulating pancreatic angiogenesis may be a useful therapeutic approach for promoting insulin delivery. Proteins synthesized by pancreatic endothelial cells could be valuable drug targets for preventing diabetic complications and progressive β cell dysfunction.

EXPERIMENTAL PROCEDURES

Mouse Strain

Due to the previously reported male sterility (Thepot et al., 2000), the *junD*-deficient colony was maintained through the breeding of heterozygous animals, which produced progeny of all genotypes in the expected ratio, ruling out any developmental defect. Genotyping was performed on DNA isolated from the tails of mice by PCR as described previously (Thepot et al., 2000). Mice were weighed and checked regularly until they died naturally. NAC (40 mM in drinking water, Sigma) was initiated during embryogenesis (via treatment of parent mice) and maintained throughout life. During all experiments, mice were maintained on standard diet containing 5% fat (#1314, Altromin). The Institut Curie ethical committee approved all experiments.

Analysis of Metabolic Parameters

Blood glucose values were determined from venous blood using an automated glucose monitor (Accu-Chek, Roche). Plasma insulin and glucagon levels were measured by the Luminex method (MENDO-75K, Linco), serum IGF-1 levels using a radioimmunoassay kit (DSL2900, Diagnostic Systems Laboratories), and ATP levels using a bioluminescence assay kit (1 699 695, Roche). Glucose tolerance tests were carried out by intraperitoneally injecting overnight-fasted mice with 2 g glucose/kg body weight. Venous blood was obtained from the tail at 0, 15, 30, 60, and 120 min after injection.

Measurement of ROS Levels

ROS levels were determined in tissue extracts using ROS-sensitive fluorescent dye (CM-H_2DCFDA, Invitrogen). Mice were killed by cervical dislocation. Immediately after dissection, pancreata were ground on a cell strainer. Medium containing 7% FCS and 10 μM CM-H_2DCFDA was poured through the strainer. Cells were then collected and incubated in medium supplemented with CM-H_2DCFDA for 30 min at 37°C in 5% CO_2 in the dark. Autofluorescence background was monitored with cells incubated in medium without CM-H_2DCFDA, which was included in each assay. Cells were loaded with DAPI (5 μM) and analyzed by fluorescence-activated cell sorting (LSR II, BD Biosciences). Fluorescence was measured on a subset of living cells characterized by DAPI-negative staining. At least 5000 events were counted within the gate parameters for each sample. Background fluorescence was subtracted from values obtained with the CM-H_2DCFDA-treated samples. Results were obtained from an average of three independent experiments for each condition, normalized with respect to measurements in control cells, and expressed as relative ROS levels.

Immunoblotting and Immunohistochemistry

Protein extracts from tissues were prepared as described in Gerald et al. (2004). Antibodies against IR (07-724, Upstate), phospho-IR (Tyr972) (07-838, Upstate), Akt (9272, Cell Signaling), phospho-Akt (Ser473) (4051, Cell Signaling), FoxO1 (ab39656, Abcam), and phospho-FoxO1 (Ser256) (ab38501, Abcam) were used.

Organs were fixed in 4% PFA for 2 hr at room temperature and then embedded in gelatin 15%/sucrose 7.5%. Ten micrometer sections were stained using antibodies against insulin (A0564, Dako), glucagon (G2654, Sigma),

HIF-1α (ab1, Abcam), VEGF-A (ab2992, Abcam), fibronectin (ab3, Oncogene), PECAM-1 (MEC 13.3, Pharmingen), SM-α-actin (A2547, Sigma), and β-Gal (G8021, Sigma).

Mouse Islet Isolation and Ca^{2+} Measurements

Islets were isolated by collagenase digestion of pancreas and hand picking (Freeman et al., 2006). Once isolated, islets were loaded for 1 hr with 1.5 μM Fura-2 AM (Molecular Probes) and incubated at 37°C in Krebs-Ringer buffer containing 115 mM NaCl, 5 mM KCl, 24 mM $NaHC0_3$, 1 mM $MgCl_2$, 0.5% BSA, 2.8 mM glucose. After loading, islets were transferred onto a coverslip and perfused continuously with 25 mM HEPES-buffered medium containing 125 mM NaCl, 5.9 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.1% BSA and supplemented with 2.8 or 16 mM glucose. Islets were imaged using a microscope with a dual excitation system. Ca^{2+} signals were obtained by rationing the fluorescence intensities detected at 510 nm after excitation at 340 nm and 380 nm. Calcium fluxes are displayed as a ratio in arbitrary units (a.u.). Background subtraction was performed by measuring fluorescence from a cell-free region in the field of view. Experiments were performed at least three times for each genotype.

Analysis of Gene Expression

Total RNA was isolated using TRIzol (Invitrogen) and treated with Turbo DNA-free (Ambion). cDNAs were prepared from 1 μg of total RNA using random hexaprimers as templates and SuperScript III (Invitrogen). Quantitative real-time RT-PCR was carried out on an ABI Prism 7500 system using SYBR green. Primer sequences are listed in Table S1.

Statistical Analysis

All experiments were performed at least three times. All data are reported as means ± SEM. Statistical analyses were performed by two-tailed Student's t test. Single, double, and triple asterisks indicate statistically significant differences: *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$. All survival analyses were carried out using the Kaplan-Meier method and the log-rank test. SPSS version 6.0 statistical software was used.

Supplemental Data

Supplemental Data include two figures and one table and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/7/2/113/DC1/.

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