Increased dosage of \textit{Ink4/Arf} protects against glucose intolerance and insulin resistance associated with aging

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Summary

Recent genome-wide association studies have linked type-2 diabetes mellitus to a genomic region in chromosome 9p21 near the \textit{Ink4/Arf} locus, which encodes tumor suppressors that are up-regulated in a variety of mammalian organs during aging. However, it is unclear whether the susceptibility to type-2 diabetes is associated with altered expression of the \textit{Ink4/Arf} locus. In the present study, we investigated the role of \textit{Ink4/Arf} in age-dependent alterations of insulin and glucose homeostasis using \textit{Super-Ink4/Arf} mice which bear an extra copy of the entire \textit{Ink4/Arf} locus. We find that, in contrast to age-matched wild-type controls, \textit{Super-Ink4/Arf} mice do not develop glucose intolerance with aging. Insulin tolerance tests demonstrated increased insulin sensitivity in \textit{Super-Ink4/Arf} compared with wild-type mice, which was accompanied by higher activation of the insulin receptor substrate (IRS)-PI3K-AKT pathway in liver, skeletal muscle and heart. Glucose uptake studies in \textit{Super-Ink4/Arf} mice showed a tendency toward increased 18F-fluorodeoxyglucose uptake in skeletal muscle compared with wild-type mice (\(P = 0.079\)). Furthermore, a positive correlation between glucose uptake and baseline glucose levels was observed in \textit{Super-Ink4/Arf} mice (\(P < 0.008\)) but not in wild-type mice. Our studies reveal a protective role of the \textit{Ink4/Arf} locus against the development of age-dependent insulin resistance and glucose intolerance.

Key words: 18F-fluorodeoxyglucose-PET; ARF; CDKN2A; CDKN2B; diabetes; insulin resistance; insulin signaling; p15\textsuperscript{ink4b}; p16\textsuperscript{ink4a}; pancreatic islet.

Introduction

Patients with type 2 diabetes mellitus (T2DM) are at higher risk for developing cardiovascular disease and have a shorter lifespan compared with the general population (Benetos et al., 2008). T2DM is increasing worldwide at alarming rates due to unhealthy lifestyles (e.g., lack of exercise, excessive caloric intake) and to population aging (Beckman et al., 2002; Nunn et al., 2009). It is well established that aged cells are more susceptible to the development of chronic diseases; however, the underlying molecular mechanisms are not completely understood (Sharpless & DePinho, 2007).

Both environmental and genetic factors play a major role in the etiopathogenesis of T2DM. Recent human genome-wide association studies and candidate gene approaches using large cohorts have linked common single nucleotide polymorphisms (SNPs) in a region of chromosome 9p21 in close vicinity to the \textit{Ink4a/Arf}/\textit{Ink4b} locus (hereby abbreviated as \textit{Ink4/Arf}) with aging-associated frailty and a variety of aging-related diseases, including coronary artery disease, myocardial infarction, stroke, and T2DM (Consortium, 2007; Melzer et al., 2008; Saxena et al., 2007; Scott et al., 2007; Sharpless & DePinho, 2007; Zeggini et al., 2007; Doria et al., 2008; Hamsten & Eriksson, 2008). The \textit{Ink4/Arf} locus is one of the main anti-oncogenic defenses of mammalian cells and its loss is among the most frequent cytogenetic events in human cancer (Gil & Peters, 2006). \textit{Ink4/Arf} encodes the tumor suppressors p16\textsuperscript{ink4a} and ARF (p14\textsuperscript{ARF} in humans, p19\textsuperscript{ARF} in mice) (CDKN2A gene) and p15\textsuperscript{ink4b} (CDKN2B gene). In humans, the locus also contains the CDKN2BAS gene, which produces a noncoding antisense RNA named ANRIL (antisense noncoding RNA in the INK4 locus) that is thought to regulate CDKN2A and CDKN2B expression (reviewed in (Cunnington & Keavney, 2011)). p16\textsuperscript{ink4a} and p15\textsuperscript{ink4b} block cell proliferation through the inhibition of CDK4,6/cyclin D kinases and the ensuing accumulation of the hypophosphorylated form of the retinoblastoma protein (Vidal & Koff, 2000).

ARF appears to play a relevant role as a pro-apoptotic factor via inhibition of MDM2, an ubiquitin ligase that destabilizes the tumor suppressor p53 (Gonzalez & Serrano, 2006). Several studies in mice and humans have demonstrated an age-dependent increase of \textit{Ink4/Arf} expression in different tissues, which might limit the regenerative potential of stem cell pools (Zindy et al., 1997; Krishnamurthy et al., 2004; Ressler et al., 2006; Collado et al., 2007). These observations have been generally interpreted as indicative of a pro-aging role for the \textit{Ink4/Arf} locus. However, various lines of evidence indicate that maximal regenerative potential may be detrimental for the long-term maintenance of stem cell pools. For example, deletion of the tumor suppressors Lkb1, Pten, Apc, p21\textsuperscript{CIP1}, weakens stem cell quiescence and results in premature exhaustion of stem cell function (Kippin et al., 2005; Zhang...
et al., 2006; Qian et al., 2008; Gan et al., 2010; Gurumurthy et al., 2010). In this context, aging-induced Ink4Arf expression could represent an anti-aging defensive response to prevent the exhaustion of stem cell pools. Indeed, Ink4Arf gain-of-function in transgenic mice confers beneficial cancer-resistant phenotypes and elicits a global anti-aging effect (Matheu et al., 2004, 2009). Moreover, p16^{INK/Arf} and p19^{Arf} expression protects from atherosclerosis development in the mouse (Gonzalez-Navarro et al., 2010; Kuo et al., 2011). In further support of a beneficial effect of the Ink4Arf locus on aging-associated diseases, in the case of the human SNP linked to atherosclerosis, the susceptibility allele is associated with lower levels of Ink4Arf expression than the normal allele (Liu et al., 2009). Thus, it remains to be established whether the Ink4Arf locus is a general pro-aging gene, a general anti-aging gene, or, alternatively, whether its effects are tissue specific.

In the present study, we investigated the impact of increased dosage of Ink4Arf on insulin and glucose homeostasis during normal physiological aging. To this end, we utilized transgenic Super-Ink4Arf mice bearing an extra allele of the entire Ink4Arf locus that behaves similarly to the endogenous gene, with minimal expression in primary embryonic cells and up-regulation in response to mitogenic overstimulation (Matheu et al., 2004). Compared with wild-type (WT) controls, aged Super-Ink4Arf mice display moderately increased levels of p16^{INK/Arf}, p15^{INK/Arf}, and p19^{Arf} in a number of tissues, yet they exhibit higher resistance to both chemically induced cancer and aging-associated spontaneous cancer (Matheu et al., 2004, 2007). In the present work, we characterized carbohydrate metabolism and β-cell function in 0.5- and 1-year-old Super-Ink4Arf mice and age-matched WT littermates. As defective signaling through insulin receptor substrate (IRS)-PI3K-AKT has been associated with T2DM and insulin resistance (Withers et al., 1998; Cho et al., 2001; Gonzalez-Navarro et al., 2008), we also examined this pathway in both groups of mice.

Results

Ink4Arf locus expression studies in Super-Ink4Arf and WT mice

To analyze the role of the locus Ink4Arf in age-associated glucose homeostasis, we first characterized gene expression in tissues relevant for carbohydrate metabolism in both transgenic Super-Ink4Arf and WT mice. Fig. 1A depicts the structure of the transgene used to generate Super-Ink4Arf, consisting of the entire Ink4Arf locus under the control of its own promoter that behaves similarly to the endogenous gene (Matheu et al., 2004). Quantification by qPCR showed a modest increase in p16^{INK/Arf}, p15^{INK/Arf}, and p19^{Arf} mRNA expression in liver of Super-Ink4Arf mice compared with WT mice, although differences were only statistically significant for p15^{INK/Arf} (Fig. 1B). In skeletal muscle, heart, and isolated islets, no significant differences in transcript levels were observed, and p19^{Arf} was not detected in skeletal muscle (Fig. 1B). Protein expression analysis also revealed a modest but not significant increase in hepatic p16^{INK/Arf}, p15^{INK/Arf}, and p19^{Arf} protein levels in Super-Ink4Arf compared with WT mice (Fig. 1C). In agreement with our qPCR studies, Western blot analysis revealed no differences in skeletal muscle, heart, and isolated islets from mice of both genotypes (Fig. 1C). Despite modest changes in the expression of the gene products of the locus, Super-Ink4Arf macrophages showed a significant increase in apoptosis compared with WT controls (Fig. 1D), demonstrating an enhanced functionality of the extra dosage of the locus in transgenic animals.

Super-Ink4Arf mice are protected against the age-associated decline of glucose homeostasis

To investigate the role of Ink4Arf in carbohydrate metabolism associated with aging, WT and Super-Ink4Arf mice were analyzed at 0.5 and 1 year of age. Body weight increased significantly with age in both WT and Super-Ink4Arf mice, and no differences were observed between genotypes (P > 0.05) at any of the time points in our study (Fig. 2A). However, while fasting plasma glucose levels were undistinguishable between 0.5-year-old WT and Super-Ink4Arf mice, glucose levels were significantly increased in 1-year-old WT mice but not in age-matched Super-Ink4Arf mice (Fig. 2B). Aging also affected differentially fasting plasma insulin levels in mice of both genotypes. Thus, no differences were observed between 0.5-year-old WT and Super-Ink4Arf mice, but insulin levels were lower in Super-Ink4Arf mice at 1 year of age (Fig. 2C). Although altered glucose homeostasis and hypercholesterolemia (Nunn et al., 2009) are often associated with aging, neither aging nor increased gene dosage of Ink4Arf affected circulating levels of total cholesterol and HDL-cholesterol (Fig. 2D, E).

We next performed glucose tolerance tests (GTT) in fasted mice. Glucose tolerance, as revealed by the area under the curve (glucose curve vs time, AUC_{glucose}), was similar in 0.5-year-old WT and Super-Ink4Arf mice (Fig. 3A). At 1 year of age, the AUC_{glucose} increased significantly in WT as compared with 0.5-year-old mice but not in Super-Ink4Arf mice (Fig. 3A). Likewise, analysis of the corresponding glucose-stimulated insulin release, expressed as AUC_{insulin}, revealed no differences between genotypes at 0.5 year of age, but we observed a significant decrease in 1-year-old Super-Ink4Arf compared with WT mice of the same age (Fig. 3B). Thus, increased Ink4Arf gene dosage prevents the development of age-dependent deterioration of glucose metabolism, but this is not accompanied by enhanced insulin secretion. To investigate whether insulin sensitivity was affected in these mice, we performed insulin tolerance tests (ITT) in 0.5- and 1-year-old mice of both genotypes (Fig. 3C). These studies revealed faster glucose disappearance in Super-Ink4Arf mice compared with WT mice after insulin injection, suggesting that increased Ink4Arf gene dosage augments insulin sensitivity.

To evaluate the effect of increased insulin sensitivity on in vivo glucose disposal and uptake, we performed 18F-fluorodeoxyglucose (18F-FDG) Positron Emission Tomography (PET)–Computer Tomography (CT) imaging studies. To this end, 1-year-old mice were intraperitonially injected with 18F-FDG and after 60 minutes of uptake, skeletal muscle was analyzed by PET–CT imaging (Fig. 4A). Quantification of the standard uptake value (SUV) showed an almost significant increase in 18F-FDG uptake in soleus muscle from Super-Ink4Arf mice compared with WT mice (Fig. 4B, P = 0.079). Furthermore, regression analysis demonstrated a positive correlation between 18F-FDG uptake and baseline glucose levels in Super-Ink4Arf mice but not in WT mice (Fig. 4C), suggesting that WT mice are insensitive to glucose levels.

Super-Ink4Arf mice exhibit increased insulin sensitivity in liver and peripheral tissues

To further characterize insulin sensitivity in age-associated glucose and insulin derangements in WT and Super-Ink4Arf mice, we analyzed insulin signaling pathway activation in vivo. Insulin exerts its actions through binding to its receptor (INS-R) and the ensuing phosphorylation of IRS at tyrosine residues (pTyr) and activation of PI3K/AKT-dependent signaling. As ablation or defective INS-R/IRS/AKT-mediated signaling in a variety of

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tissues causes major alterations in glucose homeostasis and leads to insulin resistance and T2DM (Withers et al., 1998; Cho et al., 2001; Gonzalez-Navarro et al., 2007, 2008) we analyzed IRS-AKT pathway activation in liver and peripheral tissues of 1-year-old WT and Super-Ink4/Arf mice treated with insulin. Coimmunoprecipitation experiments followed by Western blot analysis revealed reduced levels of insulin-induced pTyr-IRS1 and pTyr-IRS2 accumulation in WT compared with Super-Ink4/Arf liver (Figure 5A, B). IRS1 or IRS2 associated with the PI3K regulatory subunit p85α was similar in the liver of insulin-stimulated WT and Super-Ink4/Arf mice (Fig. 5A, B). Analysis of hepatic IRS1 and IRS2 protein and mRNA levels showed no differences between genotypes (Fig. 5C).

We next examined in peripheral tissues of 1-year-old mice the activation of AKT1/2 using a phospho-specific antibody to detect phospho-AKT1/2 (pAKT1/2). Consistent with our results in liver, we found diminished amount of insulin-stimulated pAKT1/2 in skeletal muscle and heart of WT compared with Super-Ink4/Arf mice, without

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changes in total AKT1/2 (Fig. 6). As enhanced basal activation of JNK has been associated with decreased IRS1 in insulin resistance (Hirosumi et al., 2002), we examined the phosphorylation status of this mitogen-activated protein kinase. However, the amount of pJNK was similar in control and insulin-stimulated skeletal muscle (Fig. 6A) and heart (Fig. 6B) of WT and Super-Ink4/Arf mice. Altogether, these results indicate that improved insulin sensitivity in liver and peripheral tissues contributes to protection against age-associated glucose and insulin derangements in Super-Ink4/Arf mice.

Discussion

Recent human genome-wide association studies and candidate gene strategies have linked common single nucleotide polymorphisms in a region of chromosome 9p21 near the Ink4/Arf locus with aging-associated diseases which have an important impact on public health, including coronary artery disease, myocardial infarction, stroke, and T2DM (Consortium, 2007; Melzer et al., 2007; Saxena et al., 2007; Scott et al., 2007; Sharpless & DePinho, 2007; Zeggini et al., 2007; Doria et al., 2008; Hamsten & Eriksen, 2008). However, additional work is required to define the functional consequences of these genetic variants and to ascertain whether Ink4/Arf plays a role in the development of cardiovascular disease and T2DM associated with aging. In the present study, we evaluated the impact of moderately increasing Ink4/Arf expression on glucose and insulin homeostasis during normal aging. Our results demonstrate that transgenic Super-Ink4/Arf mice carrying one intact additional copy of the Ink4/Arf locus are protected against the aging-associated alterations in glucose homeostasis normally seen in WT mice, including increased glycaemia, glucose intolerance, and insulin resistance. This protective function of Ink4/Arf gain-of-function was not associated with changes in either islet number, pancreatic β-cell area, or β-cell proliferation (Figure S1), but coincided with increased sensitivity to insulin in peripheral tissues. Our results demonstrate that insulin-stimulated activation of IRS-Pi3K-AKT-dependent signaling was greater in liver, skeletal muscle, and heart of Super-Ink4/Arf mice than in WT controls. In agreement with enhanced insulin sensitivity and signaling and lower glucose levels, Super-Ink4/Arf mice exhibited an almost significant increase in glucose uptake in skeletal muscle compared with WT mice. Furthermore, a positive correlation between glucose uptake and baseline glucose levels was observed in Super-Ink4/Arf but not in WT mice. Taken together, our results suggest that a moderate increase in Ink4/Arf function prevents age-associated derangements in glucose metabolism and insulin resistance by improving insulin-dependent activation of IRS-Pi3K-AKT signaling. In agreement with previous studies showing that Ink4/Arf expression is low in young animals and increases in several tissues during aging (reviewed in (Krishnamurthy et al., 2004)) and that Ink4/Arf expression is only moderately upregulated in tissues from 1.5-year-old Super-Ink4/Arf mice (Matheu et al., 2004), we only observed a modest increase in Ink4/Arf mRNA and protein levels in the liver of 1-year-old Super-Ink4/Arf mice (Fig. 1B, C). Importantly, however, this modest upregulation has functional consequences, as demonstrated by higher UV-induced apoptosis in Super-Ink4/Arf bone-marrow-derived macrophages (Fig. 1D).

Aging is associated with augmented susceptibility to the development of T2DM and other metabolic diseases (Rhodes, 2005), and with increased expression of Ink4/Arf in several human and mouse tissues (Zindy et al., 1997; Krishnamurthy et al., 2004; Ressler et al., 2006). In addition to promoting senescence of somatic cells, age-dependent up-regulation of Ink4/Arf might contribute to organismal aging by limiting the regenerative potential of stem cell pools (Krishnamurthy et al., 2004). Importantly, Baker et al. recently reported that clearance of p16^Ink4a^-positive senescent cells delays aging-associated disorders in the

Fig. 2 Effect of increased gene dosage of Ink4/Arf on plasmatic glucose, insulin, and cholesterol. (A) Body weight of WT and Super-Ink4/Arf mice of the indicated ages. Mice were fasted overnight to measure plasma glucose (B), insulin (C), and cholesterol (D, E). Statistical analysis was performed using one-way ANOVA.
BubR1 progeroid mouse background (Baker et al., 2008). However, evidence exists that Ink4/Arf can also elicit beneficial effects. Thus, Super-Ink4/Arf mice, which carry one extra copy of Ink4/Arf and exhibit a moderate increase in p16Ink4a, p15Ink4b, and p19Arf expression in a number of tissues at old ages, are more resistant to cancer than controls and have normal aging and lifespan (Matheu et al., 2004). Moreover, introduction of two intact additional copies of the Ink4/Arf locus in transgenic mice increases cancer resistance and extends lifespan probably by favoring quiescence and preventing unnecessary cell proliferation (Matheu et al., 2009). The Ink4/Arf locus also exerts atheroprotective functions, because increased atherosclerosis was observed in apolipoprotein-E deficient mice with global ablation of p19Arf (Gonzalez-Navarro et al., 2010) and in LDL-receptor-deficient mice with macrophage-specific deficiency of the CDKN2A gene, which encodes p16Ink4a and p19Arf (Kuo et al., 2011). The results of the present study provide additional evidence that this locus can exert beneficial effects at the organismal level beyond cancer protection.

Aging in humans and mice is associated with loss of insulin sensitivity and hyperinsulinemia (Rhodes, 2005). Moreover, impaired signaling through INS-R/IRS/AKT in a variety of tissues (including β-cells, adipocytes, leukocytes, hepatocytes) leads to insulin resistance and T2DM (Withers et al., 1998; Cho et al., 2001; Gonzalez-Navarro et al., 2007, 2008). Consistent with these results, we observed that glucose tolerance declines in 1-year-old WT but not in age-matched Super-Ink4/Arf mice. This is explained, at least in part, because insulin-mediated activation of IRS-P3K-AKT signaling in liver, skeletal muscle, and heart is enhanced as compared with age-matched control mice. This augmented signaling was accompanied by increased glucose

Fig. 3 Increased gene dosage of Ink4/Arf improves glucose tolerance and insulin sensitivity in mice. (A, B) Glucose tolerance test (GTT) was performed in 0.5- and 1-year-old WT and Super-Ink4/Arf mice. The graphs show the plasmatic levels of glucose (A) and insulin (B) at the different time points analyzed during the test. These values were used to calculate the area under the curve (AUC) (shown in the graphs in the right). (C) ITTs were performed in 0.5- and 1-year-old mice 4 h after food removal. The graphs in the left show levels of glucose at different time points analyzed during the test (relative to initial glucose level). The graph in the right depicts the AUCs. Statistical analysis was performed using one-way ANOVA.
uptake in skeletal muscle and decreased glucose levels in Super-Ink4/Arf mice. Our studies suggest that a moderate increase in Ink4/Arf function may represent a logical target for treating or delaying age-related development of insulin resistance and carbohydrate metabolism derangement.

It has been shown that HOMA-IR in patients is strongly modulated by variants in CDKN2B (Ruchat et al., 2009). However, some studies suggest that the 9p21 SNPs are associated with reduced islet function, such as decreased insulin secretion (Ruchat et al., 2009; Stancakova et al., 2009; Hribal et al., 2011), and not with differences in insulin resistance (Hribal et al., 2011) and body mass (Hotta et al., 2012). We found that moderately increased expression of the entire Ink4/Arf locus in Super-Ink4/Arf mice does not alter islet number, β-cell area, or functionality during physiological aging (Figure S1), but rather appears to protect against age-associated glucose intolerance and insulin resistance. However, previous studies have shown that deficiency in p16INK4a enhances islet proliferation and survival in mice with streptozotocin-induced β-cell ablation. Moreover, p16INK4a overexpression decreases islet proliferation suggesting that increase of p16INK4a expression might limit the regenerative capacity of β-cells (Krishnamurthy et al., 2006). It should be noted that streptozotocin treatment, a model of Type 1 diabetes, probably involves acute mitogenic stimulation and high level of p16INK4a expression, which has been shown to occur during regeneration after acute damage of a variety of tissues such as fat, skeletal muscle, hematopoietic system, nervous system, and endocrine pancreas (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006; Baker et al., 2008). However, this might not be predictive of the long-term consequences of moderately increased p16INK4a in nonacute models, such as in the studies reported here. Our observations that Super-Ink4/Arf mice do not exhibit alterations in islet number, β-cell area, or functionality during physiological aging are not unprecedented, as Kir6.2, a component of the K-ATP channels that promotes insulin secretion in β-cells, is a direct target of E2F1 (Anniccotte et al., 2009). These results suggest that both the Ink4/Arf and CDK4–PRB–E2F1 pathways regulate glucose homeostasis without affecting β-cell proliferation. Additional studies using loss-of-function and tissue-specific genetic manipulation in the mouse should address the impact of p16INK4a, p19ARF, and p15INK4B on islet function during aging in order to dissect the precise contributions of the Ink4/Arf locus to glucose homeostasis and insulin sensitivity.

### Experimental procedures

**Mice**

Care of animals was in accordance with institutional guidelines and regulations. Transgenic Super-Ink4/Arf mice used in this study carried one extra copy of the entire Ink4/Arf locus, which consisted of a 77 kb sequence that contained the complete exon structure flanked with two 20 bp short nucleotide sequences used for the identification of the transgene by PCR (Matheu et al., 2004) (Fig. 1A). Transgenic mice were obtained after 20 backcrosses into C57BL6 background and were heterozygous for the additional Ink4/Arf locus. Siblings obtained by crossing C57BL6 WT and SuperInk4Arf mice were analyzed. Mice were genotyped for the presence of the extra Ink4/Arf gene dosage using two independent PCR reactions as previously described (Matheu et al., 2004). Mice were kept on a low-fat standard diet (2.8% fat; Panlab, Barcelona, Spain) and sacrificed at 0.5 (mice between 5–7 months of age) or 1 year of age (mice between 11–13 months of age).
**Metabolic measurements**

Plasma cholesterol levels in mice fasted overnight were measured using enzymatic procedures (Wako, St. Louis, USA). HDL-cholesterol (HDL-C) was determined after precipitation of the apoB-containing lipoproteins with Heparin Calcium (Sigma, St. Louis, MI, USA) (Gonzalez-Navarro et al., 2007). For GTTs, mice received an intraperitoneal injection of glucose (2 g/Kg of body weight; Sigma) and plasma glucose and insulin levels were analyzed at different time points using a glucometer (Ascensia Elite, Bayer, Leverkusen, Germany) and ultrasensitive anti-mouse insulin ELISA (Mercodia, Uppsala, Sweden), respectively. For ITTs, mice fasted for 4 h received an intraperitoneal injection of insulin (Actrapid, Novo Nordisk, Madrid, Spain, 0.25 U/Kg of body weight), and plasma glucose levels were measured from tail blood at different time points using a glucometer.

**In vivo insulin signaling studies**

Anesthetized fasted mice (15 h) were injected with Humulina Regular (Lilly, Indianapolis, IN, USA, 0.25 U of insulin/g of BW) in the hepatic vein and killed 5 min later. Liver, skeletal muscle, and heart were removed and rapidly frozen in liquid N2 for protein extraction. Two mice per genotype were stimulated, and Western blot analysis was performed in quadruplicate.

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Fig. 5 Augmented gene dosage of Ink4/Arf increases insulin-induced signaling in the liver of 1-year-old mice. (A, B) Fasted mice were treated with insulin and sacrificed after 5 min to prepare liver protein lysates, which were subjected to immunoprecipitation with anti-IRS1 (A) or anti-IRS2 (B) antibodies and analyzed by western blot using anti-phospho-tyrosine (anti-pTyr) or anti-p85α antibodies. Representative blots are shown on the left. The graphs show relative levels of expression (average of 4 independent blots) (protein levels were relativized to unstimulated mice of the same genotype). (C) IRS1 and IRS2 protein (left) and mRNA (right) expression in liver. Liver protein lysates were subjected to immunoprecipitation with anti-IRS1 or anti-IRS2 antibodies and analyzed by western blot (WB). Representative blots are shown. The numbers below each blot show band intensity determined by densitometry after averaging 4 independent experiments (relative to WT mice). mRNA levels were normalized to cyclophilin mRNA levels and relativized to WT mouse expression.
to Western blot analysis using antibodies against phospho-AKT1/2 (pAKT1/2), to prepare skeletal muscle (A) and heart (B) protein lysates, which were subjected in 1-year-old mice. Fasted mice were treated with insulin and sacrificed after 5 min protein levels and are shown relative to unstimulated mice of the same genotype. The graphs show relative levels of expression (average of 4 independent blots). Phosphorylated (active) forms of each protein were normalized to total (Fig. 6)

In vivo PET–CT imaging to study 18F-FDG skeletal muscle uptake

Nonfasted mice were injected intraperitoneally with 11.1 MBq (300 of μCi in 0.2 mL of 0.9% NaCl) of the radiotracer 18F-FDG. After an uptake period of 60 min, mice were anesthetized (2% isofluorane) to assure immobilization during the scanning process and placed on the bed of the tomography. PET acquisition was performed during 20 min, followed by a CT scanning using a specific small animal PET CT hybrid tomograph (Albira ARS, Oncovision, Valencia, Spain) with an image resolution of <1.5 mm and a 8-cm transaxial, and a 4-cm axial field of view. The tomographic images were reconstructed by applying an ordered subset expectation maximization algorithm for the PET and a filtered back

Isolation of pancreatic islets

For islet isolation, mice were infused with Krebs buffer (127 mM NaCl, 5 mM KCl, 3 mM CaCl2, 1.5 mM MgCl2, 24 mM NaHCO3, 6 mM Heps, 2 mg/mL glucose, 0.1% albumin, equilibrated with 5% CO2 in O2). Pancreases were dissected from mice and digested with collagenase-NB8 (1 mg/mL, 17456, Serva, Heidelberg, Germany) in Krebs solution at 37 °C in a shaking water bath during 20 min. After washing with Krebs (800 g, 1 min), islets were handpicked under stereo microscope, collected (500 g, 1 min) and frozen with liquid N2 for RNA and protein analysis.

Coimmunoprecipitation and western blot analysis

Protein extracts were obtained from different tissues using a homogenizer in the presence of the ice-cold lysis TNG buffer (50 mM Tris-HCl, pH7.5, 200 mM NaCl, 1% Tween-20, 0.2% NP-40) supplemented with Complete Mini cocktail (Roche, Mannheim, Germany), 50 mM β-glycerophosphate (Sigma), 2 mM PhenylMethylSulfonflyl Fluorid (PMSF; Roche), and 200 μM Na3VO4 (Sigma).

For coimmunoprecipitation analysis, protein extracts (500 μg) were incubated 1 h 4 °C with 2 μg of rabbit polyclonal anti-IRS1 (sc-559 SANTACRUZ, Santa Cruz, CA, USA) or rabbit polyclonal anti-IRS2 (sc-8299, SANTACRUZ), followed by an incubation (1 h, 4 °C) with 30 μL of 50% Protein A/G PLUS-Agarose (sc-2003, SANTACRUZ). Beads were washed twice (16 000 g, 5 s) with TNG buffer for IRS1 immunoprecipitation or with CLB buffer (2.5 μM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1% Triton X100) for IRS2 immunoprecipitation. Immunoglobulin–protein complexes were eluted from beads by boiling in Laemmli’s buffer (30 μL) and were subjected to 8% polyacrilamide gel electrophoresis and western blot analysis.

For whole protein analysis, protein extracts (50–250 μg) were analyzed by 12% polyacrilamide gel electrophoresis (for analysis of the insulin-signaling proteins) or 15% polyacrilamide gel electrophoresis (for Ink4/Arf proteins analysis) followed by western blot (Gonzalez-Navarro et al., 2008).

The following primary antibodies were used for the western blot analysis: rabbit anti-SAPK/JNK (568G) (1/500, 9258, Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) (1/500 2994, Cell Signaling), rabbit polyclonal IgG anti-phospho-Akt (Ser473) (1/250, 9271 Cell Signaling), goat polyclonal IgG anti-Akt1/2 (1/1000, sc-1619), mouse anti-phospho-Tyr (1/500, clon 4G10, 05-321, UPSTATE, Millipore, Madrid, Spain), rabbit anti-p85α (1/500, 06-195, UPSTATE), rabbit anti-IRS2 (1/200, 06-506, UPSTATE), rabbit anti-IRS1 (1/1000, 2382 Cell Signaling), rabbit anti-p16ink4b and anti-p19Arf (1/200, sc-1207 and sc-32748, respectively, SANTACRUZ), rabbit anti-p15ink4b (1/500, 4822 Cell Signaling), and mouse anti-β-actin (1/500, A5441, Sigma). HRP conjugated secondary antibodies (1/300) from SANTACRUZ were as follows: anti-mouse IgG-HRP (sc-2031), goat anti-rabbit IgG-HRP (sc-2004), and donkey anti-goat IgG-HRP (sc-2056).

Immunocomplexes were detected with ECL Plus detection kit (GE Healthcare, ThermoFisher, Barcelona, Spain). For quantification of protein levels, we performed densitometry scanning of protein bands. Expression levels in bar graphs are shown relative to unphosphorylated images to obtain the average activity of the soleus FDG uptake (kBq/cc).

For quantification purposes, the SUV was calculated as a ratio of tissue radioactivity concentration (kBq/cc) at the time of PET acquisition and injected dose (KBq) divided by body weight (g). All these processes of visualization, coregistration and quantification were performed using the PMOD 3.0 suite (Pmod Technologies, Zurich, Switzerland).
forms of the protein or to β-actin for total protein extracts in Western blots, and to unstimulated protein levels in complementation assays.

**Gene expression analysis by quantitative real-time PCR (qPCR)**

RNA from liver, skeletal muscle, heart, and isolated pancreatic islets (50–80 islets per sample) of 1-year-old mice was obtained using TRIzol Reagent (Invitrogen, Life Technologies, Madrid, Spain) and a homogenizer. RNA purity and concentration were determined by the A260/A280 ratio, and RNA (0.5–1 μg) was retrotranscribed and amplified with SuperScript III First Strand Synthesis Platinum and SYBR Green qPCR Supermix-UDG with Rox dye (Invitrogen). Reactions were run on a thermal Cycler 7900 Fast System (liver and heart: 1 cycle of 95 °C 10 min, 40 cycles 95 °C 15 s, 60 °C 1 min; isolated islets and skeletal muscle: 1 cycle of 95 °C 10 min, 50 cycles 95 °C 15 s, 60 °C 1 min), and results were analyzed with the software provided by the manufacturer (Applied Biosystems, Life Technologies). The following primers and results were analyzed with the software provided by the manufacturer:

- **Forward (Fw):** 5′-GAGGGCGATCAGGTACTTGTG-3′
- **Reverse (Rv):** 5′-TGCCGGAGTCGACAATGAT-3′

**Apoptosis analysis in bone-marrow-derived macrophages**

Bone-marrow-derived macrophages were obtained from femoral bone marrow and differentiated (1 × 10⁶ cells/mL) 7 days in DMEM P/S, 10% fetal bovine serum and 10% L929-cell conditioned medium (as a source of macrophage colony-stimulating factor). To induce apoptosis, macrophages were exposed to ultraviolet light (80 J/m²). After 24 h, cells were trypsinized, collected by centrifugation (5 min, 400 g), fixed with 80% ethanol (30 min, −20 °C), and labeled 30 min at room temperature with propidium iodide solution (50 μg/mL, RNase A, 0.025 mg/mL). Apoptotic hypodiploid cells were detected by flow cytometry.

**Statistical analysis**

Data are presented as mean ± SEM. Differences among groups were evaluated by Student’s t-test (GraphPad Prism Software, Inc, La Jolla, CA, USA) or one-way ANOVA with Fisher’s post hoc test (Statview, SAS institute, Cary, USA). F-test was used for regression analysis. Outliers identified by Grubb’s test were not considered for quantification. Statistical significance was taken at P < 0.05.

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**Author contributions**

HG-N conceived the study, acquisition of data, performed experiments, and wrote the manuscript; AV participated in the design of study, acquisition of data, and helped in writing the manuscript; MJ participated in some experiments and revised critically the manuscript; MD designed, performed, and analyzed PET/CT imaging studies; MAP designed and analyzed PET/CT imaging studies; MS participated in the design of the study and revised critically the manuscript; DJB participated in the design of the study, supervised experimental work, and revised critically the manuscript; VA conceived the study and wrote the manuscript.

**References**


