Investigating metabolic disease in human induced pluripotent stem cells : apidocyte size, insulin signaling and hepatic lipids

Friesen, M.

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Adipocyte insulin receptor activity maintains adipose tissue mass and lifespan

Max Friesen [a, b], Carolyn S. Hudak [a], Curtis R. Warren [a], Fang Xia [a], Chad A. Cowan [a, c, d, *]

[a] Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA
[b] Department of Anatomy and Embryology, Leiden University Medical Center, 2300 RC, Leiden, The Netherlands
[c] Center for Regenerative Medicine, Massachusetts General Hospital, Boston, MA 02114, USA
[d] Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA

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ABSTRACT

Type 2 diabetes follows a well-defined progressive pathogenesis, beginning with insulin resistance in metabolic tissues such as the adipose. Intracellular signaling downstream of insulin receptor activation regulates critical metabolic functions of adipose tissue, including glucose uptake, lipogenesis, lipolysis and adipokine secretion.

Previous studies have used the *aP2* promoter to drive Cre recombinase expression in adipose tissue. Insulin receptor (IR) knockout mice created using this *aP2*-Cre strategy (FIRKO mice) were protected from obesity and glucose intolerance. Later studies demonstrated the promiscuity of the *aP2* promoter, casting doubts upon the tissue specificity of *aP2*-Cre models. It is our goal to use the increased precision of the *Adipoq* promoter to investigate adipocyte-specific IR function. Towards this end we generated an adipocyte-specific IR knockout (AIRKO) mouse using an *Adipoq*-driven Cre recombinase.

Here we report AIRKO mice are less insulin sensitive throughout life, and less glucose tolerant than wild-type (WT) littermates at the age of 16 weeks. In contrast to WT littermates, the insulin sensitivity of AIRKO mice is unaffected by age or dietary regimen. At any age, AIRKO mice are comparably insulin resistant to old or obese WT mice and have a significantly reduced lifespan. Similar results were obtained when these phenotypes were re-examined in FIRKO mice. We also found that the AIRKO mouse is protected from high-fat diet-induced weight gain, corresponding with a 90% reduction in tissue weight of major adipose depots compared to WT littermates. Adipose tissue mass reduction is accompanied by hepatomegaly and increased hepatic steatosis.

These data indicate that adipocyte IR function is crucial to systemic energy metabolism and has profound effects on adiposity, hepatic homeostasis and lifespan.

1. Introduction

Insulin resistance has reached epidemic levels, contributing to an increase in the prevalence of obesity, type 2 diabetes (T2D), and cardiovascular disease [1]. The onset of insulin resistance in metabolic tissues precipitates a cascade of pathologies, culminating in failure of glucose homeostasis and exhaustion of insulin-producing pancreatic beta cells. Insulin signaling through the insulin receptor (IR) plays a pivotal role in the differentiation, development and cellular function of both white and brown adipose tissue. Not only does the IR regulate lipid metabolism through suppression of lipolysis and induction of lipogenesis in adipocytes, it also stimulates glucose uptake [2]. Adipose tissue regulates systemic energy metabolism by secreting hormones, called adipokines. This endocrine function of adipose tissue is in part regulated by insulin through the IR signaling pathway [3]. Adipocyte insulin resistance can affect each of these functional capacities, with adverse consequences for systemic metabolic health [4].

Previous studies have examined the role of insulin signaling in the adipose tissue, utilizing a “fat-specific” insulin receptor knockout (FIRKO) mouse, generated using an *aP2* promoter-driven Cre recombinase [5]. Fat mass was reduced in FIRKO mice, and

* Corresponding author. Cowan Laboratory, Harvard University, Department of Stem Cell & Regenerative Biology, Sherman Fairchild Bldg, Rm 101-23, 7 Divinity Ave, Cambridge, MA 02138, USA.
E-mail addresses: maxfriesen@gmail.com (M. Friesen), chadacowan@gmail.com (C.A. Cowan).

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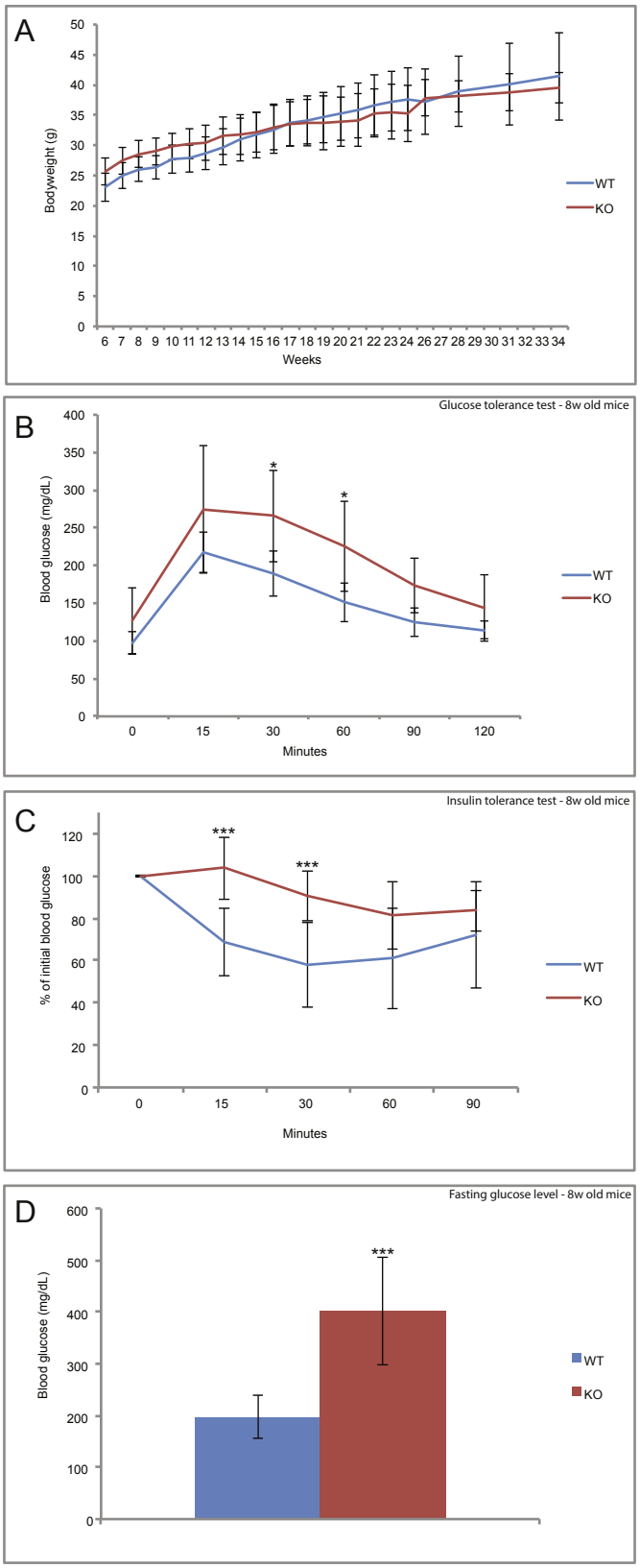


Fig. 1. Characterizing the AIRKO mouse. A) Bodyweight measurements over time from AIRKO and WT littermates. B) GTT performed on 2-month-old mice. C) ITT performed on 2-month-old mice. D) Fasting blood glucose of 4-h fasted 2-month-old mice. Mouse groups N = 13 WT mice and 11 KO mice. Error bars, standard deviation. * = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001.

these mice were protected from obesity and associated glucose intolerance FIRKO mice have an increased median and maximum life span, presumably as a product of protection from obesity and glucose intolerance [6].

One drawback of these studies was that the authors employed an *aP2* promoter-driven Cre. Since publication of the FIRKO mouse studies, other groups have demonstrated the promiscuity of the *aP2* promoter, driving expression of transgenes in several off-target tissues [7–10]. As the *aP2* promoter driven Cre is not adipose tissue specific, this can lead to confounding results, as phenotypes may be the product of knockout in other tissues.

The reported phenotypes of the FIRKO mouse were surprising, given the importance of the IR in adipose tissue function. We re-examined the phenotypes of FIRKO mice and to investigate this in further depth, we use the *Adipoq* promoter-driven Cre transgene. *Adipoq* expression is restricted to mature fat, mitigating effects of IR knockout on adipogenesis [11]. The resulting Adipocyte-specific Insulin Receptor Knockout (AIRKO) and FIRKO mice are protected from the exacerbation of glucose intolerance by age and obesity. They do suffer from intrinsic metabolic defects. Adipose tissue mass is significantly reduced, and compensation for the lack of adipose tissue results in hepatomegaly and increased triglyceride accumulation in the liver. AIRKO and FIRKO mouse lifespan is significantly reduced, indicating that adipose IR activity is critical for extending lifespan by promoting systemic metabolic health.

2. Materials and methods

2.1. Animals and diets

The Harvard University IACUC approved all protocols. IRLox mice were bred with transgenic mice that express Cre recombinase driven by the adipose-specific Adipoq promoter/enhancer region (AIRKO mice), or the aP2 enhancer (FIRKO mice). AIRKO and FIRKO mice were obtained with the expected Mendelian frequency and displayed normal development. All mouse strains were acquired from The Jackson Laboratory. Mice were housed on a 12 h light/dark cycle with ad libitum access to water and food. For high-fat diet (HFD) experiments the mice were fed chow diet until 10 weeks of age, and at that point transferred to a 60% high fat diet (Harlan Teklad) for an additional 14 weeks.

2.2. Glucose and insulin tolerance tests

Glucose tolerance tests (GTT) were performed on overnight-fasted mice injected intraperitoneally (IP) with 1 g of D-glucose per kg of bodyweight. Insulin tolerance tests (ITT) were performed on 4-h fasted mice injected IP with 1 IU/kg bodyweight of insulin (Sigma). Pyruvate tolerance tests (PTT) were performed on overnight-fasted mice injected IP with 1 g of pyruvate per kg of bodyweight. During GTT, ITT, and PTT, blood glucose levels were measured using the OneTouch Ultra 2 system (OneTouch) at 0, 15, 30, 60, and 90 min post-injection. During GTT and PTT, blood glucose was also measured at 120 min.

2.3. Tissue analysis

Tissues were dissected, photographed, weighed and fixed in 4% paraformaldehyde (Electron Microscopy Sciences). Tissues were paraffinized and cut into sections by the Harvard Stem Cell and Regenerative Biology Histology-Immunohistochemistry core laboratory using the method described in Ref. [12]. Sections were stained with hematoxylin and eosin [13] and imaged on a BX53 microscope (Olympus).

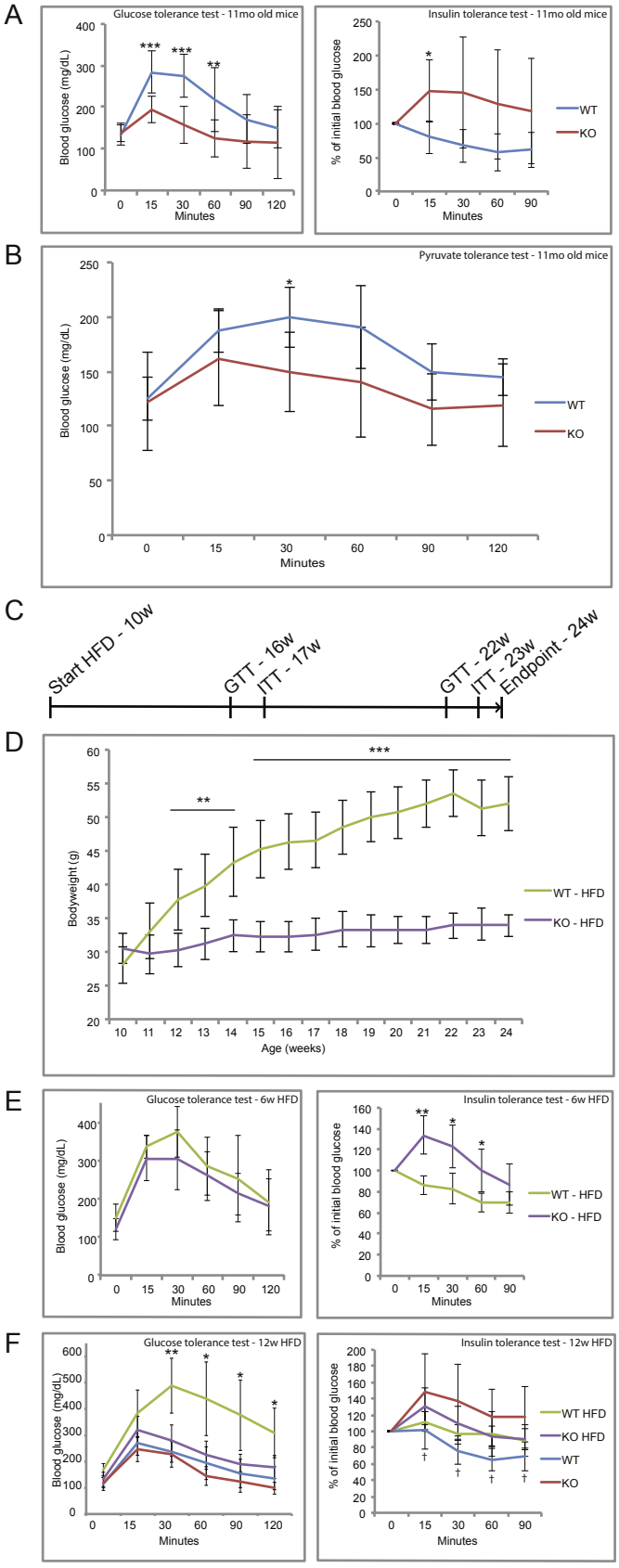


Fig. 2. AIRKO mice are protected from age- and HFD-related metabolic deficiencies. A) GTT and ITT performed on 11-month-old AIRKO mice and WT littermates. B) PTT performed on 11-month-old mice. C) Schematic showing the experimental design of the HFD study. D) Bodyweight measurements over time from the AIRKO and WT groups on HFD. E) GTT and ITT performed on 16 week old mice after 6 weeks on HFD. F) GTT and ITT performed on 6 month old AIRKO and WT mice on chow or HFD as indicated. Mouse groups N = 10 WT and 6 KO for the age-study, N = 6 WT and 7 KO for the HFD study, N = 13 WT and 11 KO for the 6 month old chow cohort. Error bars, standard deviation. * = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001, † = p-value ≤ 0.05 of WT versus other groups.

2.4. Statistical analysis

All data points are represented as mean ± standard deviation and analyzed by unpaired two-tailed Student's t-test. Statistical significance is shown by * = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001.

3. Results

3.1. AIRKO mouse characterization

AIRKO mice exhibit normal growth, as evidenced by unaltered bodyweight compared to WT controls (Fig. 1A). AIRKO mice do not develop gross obesity or severe lipodystrophy as a consequence of abrogated adipose IR activity. Upon dissection, AIRKO mice were found to have a reduction in adipose tissue mass whereas wild-type (WT) mice displayed normal adipose depots. To assess the effect of IR deletion in mature adipocytes we performed a glucose tolerance test (GTT) on 2-month-old mice. The AIRKO group displayed significantly higher blood glucose until 90 min after glucose administration (Fig. 1B). Insulin tolerance tests (ITT) revealed a significant reduction in insulin responsiveness (Fig. 1C). There is also significant fasting hyperglycemia after a 4-h fast before administration of the ITT (Fig. 1D). This presents a clear dysregulation of glucose and insulin metabolism, despite equivalent body weight.

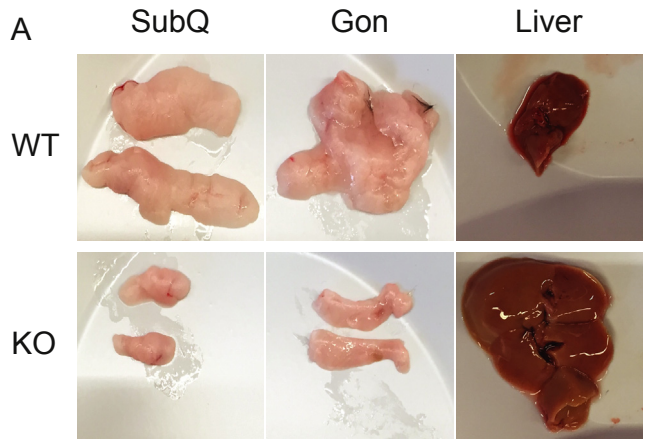
3.2. The AIRKO mouse is protected from age- and diet-related metabolic deterioration

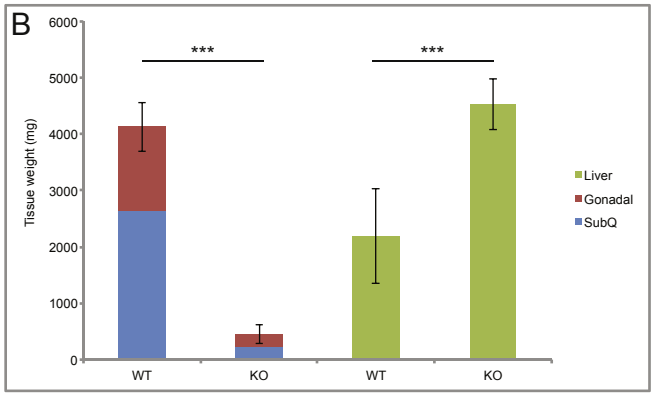
Mice acquire insulin resistance with age, leading to dysregulated glucose and lipid metabolism [14]. After finding reduced glucose tolerance and insulin sensitivity in 2-month-old mice, we decided to evaluate metabolic competency of aged AIRKO mice. A GTT of 11-month-old AIRKO mice revealed significantly higher glucose tolerance than the WT littermate control group, which acquired age-related glucose metabolism deficiencies (Fig. 2A). Despite this shift in glucose metabolism, the AIRKO group remains insulin resistant with a reduced response to insulin injection compared to WT controls (Fig. 2A). Fasting hyperglycemia in 11-month-old mice is consistent with findings in two-month-old mice (data not shown).

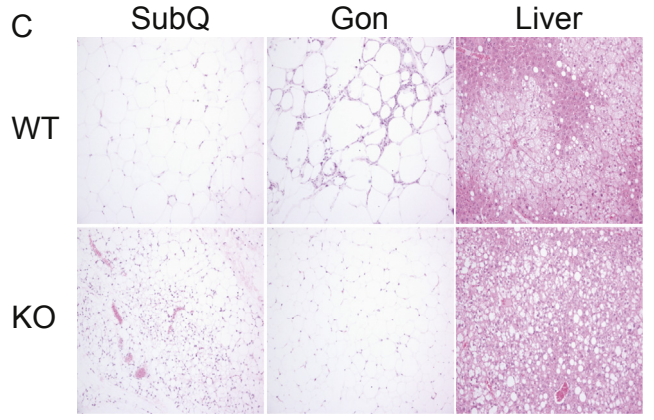
To investigate effects of adipose IR ablation on systemic non-adipose metabolic health, we studied hepatic gluconeogenesis using a pyruvate tolerance test (PTT). AIRKO mice display significantly less elevated blood glucose 30 min after the pyruvate bolus (Fig. 2B).

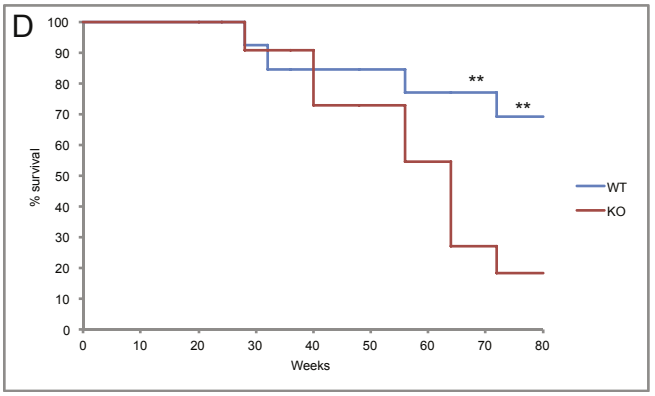
3.3. High-fat diet effects on AIRKO mouse metabolism

After assessing the effect of age-related insulin resistance on this mouse model, we subjected the AIRKO mouse to HFD, which is a standard mouse model of diet-induced obesity and associated metabolic effects. During the HFD regimen we performed GTTs and ITTs and regularly weighed the mice to monitor the metabolic health and the progression of obesity (Fig. 2C). AIRKO mice resisted weight gain while the WT littermates gained a statistically









significant amount of mass (Fig. 2D). The WT mice suffer glucose intolerance as a consequence of HFD bringing them to parity with the AIRKO mouse after 6 weeks of HFD feeding (Fig. 2E). Despite this shift in relative glucose tolerance KO mice remain significantly insulin resistant after 7 weeks of HFD feeding, when compared to WT littermates (Fig. 2E). After 12 weeks of HFD administration, glucose sensitivity of AIRKO mice remained unchanged. In contrast, WT mouse glucose clearance worsened after 12 weeks of HFD, and was significantly lower than that of the AIRKO mice (Fig. 2F). After 13 weeks of the HFD regimen, the insulin sensitivity of WT mice has diminished to match that of AIRKO mice, regardless of the AIRKO dietary regimen (Fig. 2F).

3.4. Adipocyte IR expression is necessary for adipose tissue expansion and lifespan

Liver, subcutaneous adipose, and gonadal adipose tissue samples were collected and weighed at the end of the HFD regimen. Both fat depots were greatly reduced in mass in the AIRKO group, while the liver displayed clear enlargement (Fig. 3A). There was a 90% reduction in adipose tissue weight, and a doubling of the liver weight in AIRKO mice compared to WT controls (Fig. 3B). Cellular morphology corroborated this finding, as the adipocytes of the AIRKO mouse have almost no lipid droplets in the case of subcutaneous adipose, and reduced lipid droplet size in gonadal fat. Lipid accumulation is increased in AIRKO hepatocytes when compared to the WT sections (Fig. 3C).

In spite of the aforementioned enhanced glucose metabolism during aging relative to the control group, AIRKO mice have a significantly reduced median and maximum lifespan (Fig. 3D). This communicates a significant health burden conferred by the metabolic disadvantages of the AIRKO mouse, despite the buffering of age-related deterioration of insulin sensitivity and glucose tolerance.

3.5. Two month-old FIRKO mice are insulin insensitive

Previous reports have found metabolic and survival benefits from knocking out the IR in adipocytes (the FIRKO mouse) using the aP2 promoter-driven Cre recombinase [5,6]. We replicated the FIRKO mouse study to directly compare this model with the AIRKO mouse. FIRKO mouse fat pads were of insufficient mass for protein extraction, thereby confirming functional knockout and mirroring the phenotype of the AIRKO mouse. There is no bodyweight difference between FIRKO mice and WT littermates (Fig. 4A). In our hands FIRKO mice demonstrate hyperglycemia after an overnight fast, but are equally adept at clearing the injected glucose bolus as the control group when examined at two months old when subjected to a GTT (Fig. 4B). FIRKO mice are insulin resistant compared to WT littermates at two months of age as determined by ITT (Fig. 4B). When aged to six months, the FIRKO mouse no longer displays fasting hyperglycemia relative to WT and has normalized its insulin sensitivity to the level of the WT mouse (Fig. 4C). These phenotypes emulate the AIRKO mouse.

Fig. 3. IR knockout in adipocytes affects lipid accumulation and survival. A) Representative pictures of dissected subcutaneous (SubQ) and gonadal (Gon) adipose tissue as well as liver. B) Quantified weight of tissues shown in 3 A. C) Histology slides of dissected subcutaneous (SubQ) and gonadal (Gon) adipose tissue as well as liver. Images are taken at 20x zoom. D) Kaplan-Meier plot of the AIRKO mouse versus WT littermates. Mouse groups N = 6 WT and 7 KO for the HFD study, N = 13 WT and 11 KO for the start of the Kaplan-Meier plot. Error bars, standard deviation. * = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001.

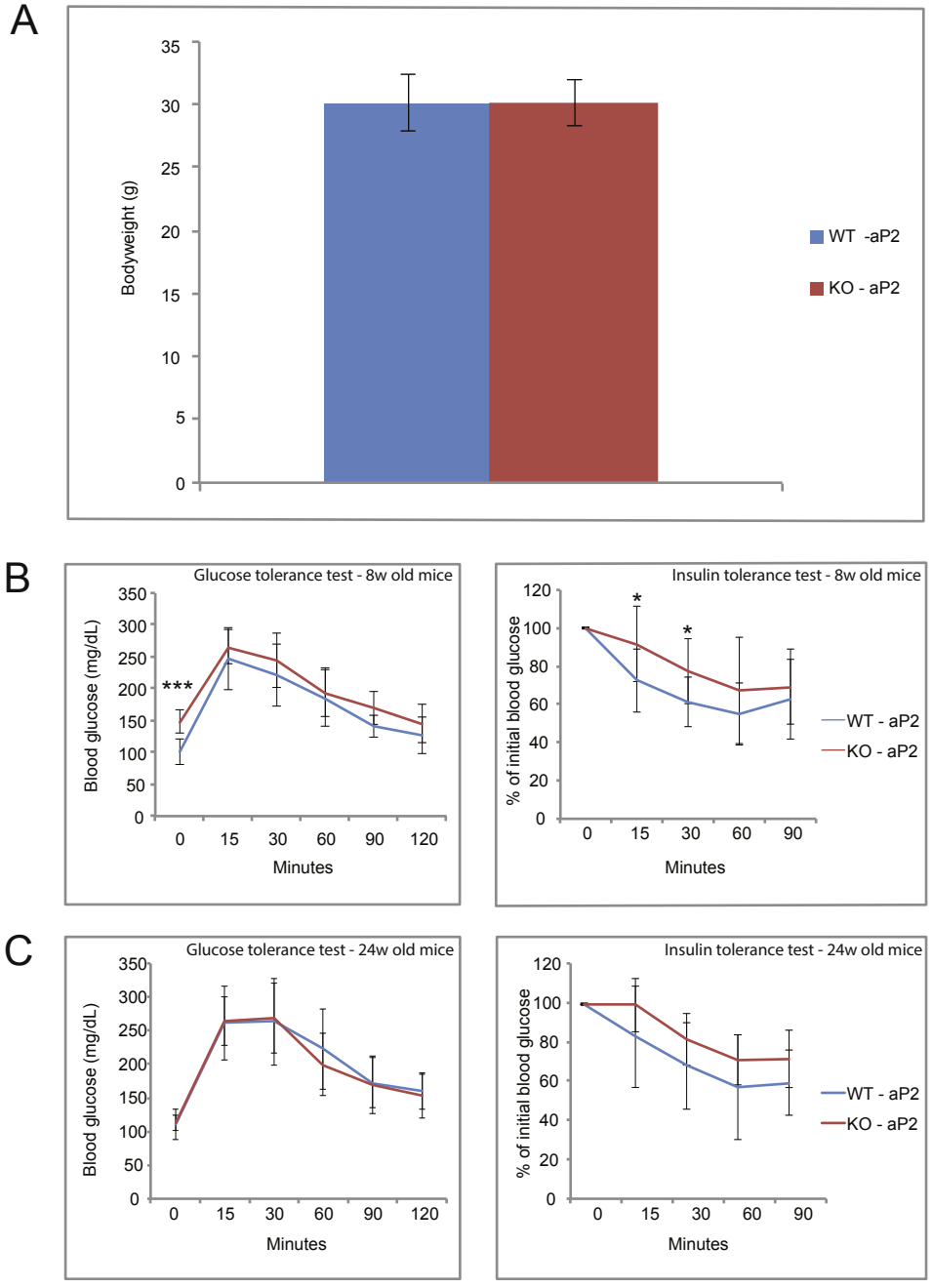


Fig. 4. The FIRKO mouse is insulin resistant at two months of age. A) Bodyweight of FIRKO and WT mice at 4 months old. B) GTT and ITT performed on 2-month-old mice. C) GTT and ITT performed on 6-month-old mice. Mouse groups N = 10 WT and 10 KO. Error bars, standard deviation. * = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001.

4. Discussion

In the present report we demonstrate the effects of adipocyte-specific deletion of the insulin receptor. This AIRKO model is protected from age-associated dysregulation of glucose metabolism, as well as obesity-induced insulin resistance. At two- and eleven-months of age AIRKO mice have identical blood glucose profiles during a GTT, exhibiting protection from age-related metabolic decline. AIRKO mice have identical weights on chow or HFD, as their adipose tissues do not expand in response to caloric excess. This suggests ablation of IR signaling in adipocytes prevents these mice from storing excess dietary fat in the adipose tissue. After HFD treatment this deficiency leads to a 90% reduction in fat mass, while adipose is practically absent on a chow diet. This demonstrates the necessity of the insulin receptor for adipose tissue development and function.

Despite the relative protection from age- and diet-induced glucose intolerance, young AIRKO mice are glucose intolerant and insulin resistant compared to age-matched WT mice. AIRKO mouse insulin resistance at two months of age is equal in severity to the effects of 12 weeks of HFD-induced obesity or age-acquired insulin resistance on insulin insensitivity in WT mice. AIRKO mice also present with fasting hyperglycemia, divulging an obvious inadequacy in basal non-challenged glucose clearance. These

observations highlight the central role of adipose tissue insulin signaling in systemic glucose and insulin homeostasis. Disrupting this axis in AIRKO mice led to severe dysregulation of glucose homeostasis. These metabolic deficiencies resulted in a significant decrease in median and maximum lifespan in chow-fed AIRKO mice.

After 11 months of age, AIRKO mice cannot convert pyruvate to glucose during a PTT, indicating impairment of hepatic gluconeogenesis. This exhaustion of the liver's capabilities is likely due to increased hepatic triglyceride storage and resultant non-alcoholic fatty liver disease (NAFLD). The diagnosis of NAFLD is corroborated by the doubling of liver size of the AIRKO mice on HFD. Furthermore, triglyceride accumulation is increased in AIRKO livers (as evidenced by immunohistochemistry) as a consequence of decreased adiposity. This reveals that while abrogated insulin signaling in the adipose reduces the accumulation of fat in those depots, the liver now becomes the primary reservoir for triglyceride storage. The liver is a last resort to maintain lipid homeostasis in the absence of adipose tissue, but accumulation of excess hepatic lipids affects insulin insensitivity and diminishes metabolic functionality of the liver [15]. It is probable that skeletal muscle accumulates ectopic lipids in the AIRKO model as well, contributing to diminished insulin-stimulated glucose uptake.

Previous reports on the FIRKO model have shown beneficial health effects in these mice including extended lifespan and favorable effects on glucose clearance [5,6]. These results were directionally inconsistent with the phenotype of the AIRKO mouse. The FIRKO model exhibits more favorable glucose and insulin response profiles when compared with the deleterious AIRKO phenotype, but in our hands, the *aP2* promoter-driven IR knockout does not confer any positive effects on glucose clearance when compared to WT controls.

In the AIRKO model, insulin resistance is partly decoupled from glucose sensitivity. This implies there is some adipose function independent of systemic insulin resistance, which regulates glucose homeostasis. This adipose function is affected by HFD and age, resulting in significant glucose intolerance and insulin insensitivity in WT mice. The AIRKO mice are protected from both of these metabolic stressors. We speculate that this is due to the reduction in adipose depot size of the AIRKO mouse. In this model a reduction in adipocyte number leads to a decrease in adipokine secretion, which may mediate the systemic phenotypes we show. A number of adipokines exert control over glucose clearance in this context [16,17]. The delicate interplay between adipocyte insulin signaling, adipose depot mass, and aging remains to be further investigated. Inquiry into these interactions in the AIRKO model may identify therapeutic targets for the treatment of insulin resistance.

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Transparency document

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