Differential Roles of Insulin and IGF-1 Receptors in Adipose Tissue Development and Function

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Running title: Deletion of IR in fat causes lipoatrophic diabetes.
SUMMARY

To determine the roles of insulin and IGF-1 action in adipose tissue we created mice lacking either the insulin receptor (IR), IGF-1 receptor (IGF1R), or both using Cre-recombinase driven by the adiponectin promoter. Mice lacking IGF1R only (F-IGFRKO) had a ~25% reduction in white and brown adipose tissue (WAT and BAT), whereas mice lacking both IR and IGF1R (F-IR/IGFRKO) showed an almost complete absence of WAT and BAT. Interestingly, mice lacking only the IR (F-IRKO) had a 95% reduction in WAT, but a paradoxical 50% increase in BAT with accumulation of large unilocular lipid droplets. Both F-IRKO and F-IR/IGFRKO mice were unable to maintain body temperature in the cold and developed severe diabetes, ectopic lipid accumulation in liver and muscle, and pancreatic islet hyperplasia. Leptin treatment normalized blood glucose levels in both groups. Glucose levels also improved spontaneously by 1 year of age, despite sustained lipodystrophy and insulin resistance. Thus, loss of IR is sufficient to disrupt white fat formation, but not brown fat formation and/or maintenance, although it is required for normal BAT function and temperature homeostasis. IGF1R has only a modest contribution to both WAT and BAT formation and function.
INTRODUCTION

Insulin and insulin-like growth factor 1 (IGF-1) play important roles in the development and differentiation of white and brown adipose tissue (WAT and BAT) (1; 2). These hormones act through insulin and IGF-1 receptors (IR and IGF1R), which are highly homologous and share many overlapping downstream signaling pathways. Furthermore, while insulin and IGF-1 bind with higher affinity to their cognate receptors, insulin can also bind and activate the IGF1R and vice versa (3; 4). In preadipocytes, IGF1R expression is higher than IR expression, whereas in mature adipocytes the opposite is true (3; 5). Fat-specific deletion of IR results in reduced WAT and BAT mass (6; 7), whereas mice with a fat-specific deletion of IGF1R alone have been reported to have slightly increased adipose tissue mass associated with increased overall body growth (8). Deletion of both receptors in fat leads to a marked reduction in WAT and BAT mass and obesity resistance, even when challenged with a high fat diet (9).

One limitation of many of these previous studies is that conditional inactivation of the receptors was achieved using a targeting approach based on the expression of the Cre-recombinase under the control of aP2 promoter. This can lead to a variable degree of recombination efficiency in different fat depots, as well as potentially important off-target recombination events (10-13). In the present study, we deleted the IR and/or IGF1R specifically in adipose tissues using the adiponectin-Cre (Adipo-Cre) transgene, which produces more uniform and efficient deletion, and is completely adipocyte-specific (10-13). Here we show that in white adipose tissue, the IGF1R plays only a modest role, whereas mice lacking IR alone or both IR and IGF1R display a lipodystrophic phenotype with severe diabetes, insulin resistance and ectopic fat distribution in both muscle and
liver. BAT mass, on the other hand, is differentially regulated and is only decreased when both IR and IGF1R are absent, thus indicating a more integrated role of these receptors in brown adipose tissue.
MATERIAL AND METHODS

Animals and diets

All protocols were approved by the IACUC of the Joslin Diabetes Center and were in accordance with NIH guidelines. Mice were housed at 20-22°C on a 12 h light/dark cycle with ad libitum access to water and food (Mouse Diet 9F, PharmaServ). Fat-specific IR, IGFR, and IR/IGFR knockout mice were generated by breeding IR\textsuperscript{lox/lox} and/or IGFR\textsuperscript{lox/lox} mice on a C57Bl/6 – 129Sv genetic background (9) with mice carrying Cre recombinase driven by the adiponectin promoter (Adipo-Cre) on a C57Bl/6 background (12). IR/IGFR\textsuperscript{lox/+} heterozygous mice were bred to generate Adipo-Cre homozygous littermate mice for all three genotypes. Adipo-Cre positive males and Adipo-Cre negative female mice of each genotype were used for breeding, and breeder pairs of each genotype were replaced simultaneously every 6 months to ensure that there is little or no genetic drift. Male mice were used throughout the study, and control (Adipo-Cre negative floxed) mice from all 3 genotypes were pooled into a single control group, since none demonstrated physiological abnormalities.

Insulin tolerance test and leptin treatment

Insulin tolerance tests were performed after a 2h fast with 1.25U of insulin/kg for control and F-IGFRKO mice and 2 units/kg for F-IRKO and F-IR/IGFRKO mice (Humulin R, Lilly). Glucose levels were measured using a glucose meter (Infinity, US Diagnostics). Leptin (10 µg per mouse per day, Sigma) or saline were administered using osmotic pumps (Alzet Model 1002) inserted subcutaneously in 3 month-old mice for 14 days.

Serum analyses
Serum parameters were determined by the Joslin’s DERC assay core using commercial kits. Hormones were assessed by ELISA, adipokines were measured by a multiplex assay and triglycerides, cholesterol, FFA were assessed by colorimetric assays.

**Body temperature and cold exposure**

Body temperature was measured in 3 month-old mice using a RET-3 rectal probe (Physitemp). For cold exposure, mice were housed individually at an ambient temperature of 6°C. Temperature was measured every 30 min for 3 hours or until body temperature dropped below 25°C.

**Measurement of beta cell proliferation and islet area**

Pancreatic tissue was immunostained using anti-Ki67 (BD) and anti-insulin (Abcam) antibodies. Ki67+ beta cells were visualized by immunofluorescence microscopy and counted by a blinded observer (14). Insulin positive cells co-localized with nuclear DAPI and Ki67 immunostaining were counted as proliferating beta cells. Beta cell area was determined by image J software (NIH) and calculated as insulin positive area divided by total pancreas area.

**Gene expression analysis**

mRNA extraction and quantification was performed as previously described (9)

**Protein extraction and immunoblot analysis**

Muscle tissue was frozen, pulverized, and homogenized in RIPA buffer (Millipore) with protease and phosphatase 2 and 3 inhibitors (Sigma). Lysates were subjected to SDS-PAGE, transferred to PVDF membranes (Thermo Scientific) and blotted using insulin receptor beta (Santa Cruz), Glut4 (Chemicon), or β-actin-HRP
(Santa Cruz) antibodies. Quantification of immunoblots was performed using ImageJ.

**Histology and muscle fiber size**

Frozen cross-sections of TA muscle were fixed in 4% paraformaldehyde for 5 minutes, washed, permeabilized with 0.5% triton-X100 and immunofluorescently stained for laminin (Sigma L9393, 1:200 dilution) with Alexa Fluor 488-conjugated secondary antibodies (Life Technologies, 1:500). Images of laminin-stained fibers were taken in the same superficial area of TA muscles then changed to grayscale using Adobe Photoshop (v6.0). ImageJ software was used to threshold images and the "Analyze Particles" tool was used to find all cross sections between 500 and 1,000,000 square pixels with circularity of 0.5-1.0. Pixel area was converted to $\mu m^2$ by quantifying the length of the 200 $\mu m$ scale bar in pixels.

**Muscle lipid accumulation, succinate dehydrogenase (SDH) and Nile Red co-staining.**

Cross-sections of TA muscle were stained for SDH as previously described (15). After SDH staining, sections were rinsed in ice-cold PBS, fixed in 4% paraformaldehyde and washed with PBS. Sections were then incubated in a 1:10,000 dilution of 1 mg/ml Nile Red (ThermoFisher Scientific, N-1142), washed twice with PBS and mounted with SlowFade Gold with DAPI (Life Technologies S36938). Skeletal muscle and heart triglycerides were quantified as previously described (16).

**Mitochondrial nuclear to DNA ratio and oxygen consumption.**

Mitochondrial DNA content was assessed by qPCR (Applied Biosystems 7900) using primers for five different mitochondrial encoded genes (ND1, ND4,
cyclooxygenase 2 and 3 and rRNA) and two different nuclear encoded genes (Chdh and Actb). Results are expressed as an average of the different mitochondrial to nuclear DNA ratios.

Brown adipose tissue oxygen consumption rate was assessed using freshly isolated mouse BAT as previously described (17). Briefly, BAT was rinsed with unbuffered KHB media, cut into pieces (~10 mg), and washed extensively. Each piece was placed in a single well of a XF24-well Islet Flux plate (#101174-100; Seahorse Bioscience, North Billerica MA) and analyzed in a Seahorse XF24 Analyzer. Each OCR value was an average of 5 independent pieces per tissue per experiment and adjusted to total DNA content.

**Statistical analyses**

All data are presented as mean ± SEM and analyzed by unpaired two-tailed Student’s t-test or analysis of variance (ANOVA) as appropriate.
RESULTS

**Generalized lipodystrophy in mice lacking IR in the fat**

Mice with a homozygous fat-specific knockout of either the IR, IGF1R or both were generated by breeding mice with IR and IGF1R floxed alleles (9) and mice carrying a Cre-recombinase transgene driven by the adiponectin promoter (12). By three months of age, male F-IGFRKO mice displayed a ~25% decrease in subcutaneous (inguinal) and visceral (epididymal) WAT weight, whereas F-IRKO mice displayed a >90% reduction in weights of both WAT depots (Figure 1A). Visceral WAT in F-IRKO mice displayed dramatically reduced cell size, whereas the residual subcutaneous WAT was a mixture of connective tissue, undifferentiated cells, and a few lipid-laden adipocytes (Figure 1B). The fibroblast-like cells could be preadipocytes or dedifferentiated adipocytes, or even dying differentiated adipocytes, or just be a residue of the stromavascular fraction of a normal adipose tissue depot. Mice with knockout of both IR and IGF1R had no apparent visceral WAT and a 99% reduction in subcutaneous WAT with few identifiable adipocytes (Figure 1A, B).

Consistent with the reduction in fat mass, circulating leptin, resistin and adiponectin levels were markedly decreased in F-IRKO and F-IR/IGFRKO mice (Figure 1C). Despite its normal appearance, F-IGFRKO WAT displayed 30-50% decreases in *Glut4, Fas, Hsl, leptin* and *adiponectin* mRNA (Figure 1D). These genes were more robustly decreased in F-IRKO WAT, which also exhibited reduced mRNA levels of *Atgl* (50%), *resistin* (35%), and the adipocyte markers *C/EBPa, Pparγ* and *aP2* (40%), indicating an impairment of differentiation, an impairment in the maintenance of the fully differentiated adipocyte transcriptional program, or changes in cellular composition.
of the depot (Figure 1D). Thus, while both IR and IGF1R are necessary for normal adipocyte gene expression, IR is more critical than IGF1R in maintenance of WAT mass, gene expression and adipokine levels.

**Differential role of IR versus IGF1R in BAT development and function**

As in WAT, the IGF1R plays a modest role in BAT formation with a 25% reduction in BAT mass and normal histology in F-IGFRKO mice (Figures 2A, B). By contrast, F-IRKO mice displayed a paradoxical 50% increase in BAT mass with many cells containing unilocular lipid droplets, resembling white adipocytes (Figure 2A,B). However, when both receptors were eliminated, there was a >85% reduction of BAT mass, and the remaining BAT was composed of a mixture of undifferentiated cells, small brown adipocytes and some large unilocular fat cells (Figure 2B).

Despite the white fat-like appearance of F-IRKO BAT, gene expression markers of white fat, such as *leptin* and *Hoxc8* were not increased, nor was there a decrease in markers of brown fat, such as *Zic1* and *Lhx8*, or beige fat, such as *Tbx1* (Figure 2C). Brown fat in F-IR/IGFRKO mice, on the other hand, displayed decreases in *leptin* (60%) and *Hoxc8* (80%) and 4- to 33-fold increases in *Zic1*, *Lhx8* and *Tbx1*. Expression of genes involved in BAT function, such as *Ucp1*, *Prdm16*, *Elovl3*, *Cidea*, *Dio2*, *Adrb3* and *Tfam* was reduced by 40 to 98% in F-IR/IGFRKO and F-IRKO BAT (Figure 2D). Likewise *Pparγ*, *Hsl*, *Glut4* and *Fas* were decreased by 30% to 99% (Figure 2E). In BAT lacking only the IGF-1 receptor, *Hsl*, *Fas* and *Glut4* mRNAs were modestly reduced, but these did not reach statistical significance (Figure 2E).
Consistent with the altered BAT mass, morphology and gene expression, both F-IRKO and F-IR/IGFRKO mice were unable to maintain body temperature when placed at 6°C for three hours; F-IRKO mice dropped their core body temperature by 5°C and F-IR/IGFRKO mice by >12°C (Figure 2F). While the lack of insulating subcutaneous adipose tissue or some change in shivering could contribute to the cold sensitivity of F-IRKO and F-IR/IGFRKO mice, expression of *Ucp1, Pgc1α, Elovl3, Cidea, Tfam* and *Adrb3* were consistently lower in F-IRKO BAT compared to controls, even after cold exposure (Figure 2G). Furthermore, F-IRKO BAT had decreased mitochondrial content and decreased oxygen consumption (Figure 2H). F-IGFRKO mice, on the other hand, were cold-tolerant and had normal expression of thermogenic genes in BAT. These results indicate that while IGF-1 receptor is required for development of normal BAT mass, it is not essential for normal thermogenic function. The insulin receptor, on the other hand, is dispensable for BAT development, but essential for the thermogenic function of BAT.

**F-IRKO and F-IR/IGFRKO mice develop diabetes which is reversed by leptin replacement.**

Both F-IRKO and F-IR/IGFRKO mice developed overt diabetes with random-fed blood glucose levels of ~500 mg/dl, and 9- to 12-fold increases in insulin levels at 3 months of age (Figure 3A and B). Likewise, F-IRKO and F-IR/IGFRKO mice were highly insulin resistant by insulin tolerance testing (ITT), even at doses of insulin of 2 U/kg (Figure 3C). By contrast, F-IGFRKO mice had normal glucose and insulin levels, as well as normal ITTs. F-IRKO and F-IR/IGFRKO mice also developed significant
dyslipidemia with increased circulating triglycerides (TG) and cholesterol levels, while free fatty acids (FFA) were increased in F-IR/IGFRKO mice only (Figure 3D). Dyslipidemia was not observed in F-IGFRKO mice.

Leptin treatment has been shown to reverse hyperglycemia in mice and humans with lipoatrophic diabetes (18-21). In F-IRKO and F-IR/IGFRKO mice, leptin administration by subcutaneous infusion pumps results in a significant decrease in glucose levels within 4 days and almost complete normalization by 2 weeks (Figure 3E). Leptin-treated mice also exhibited a 2-3 g weight loss during treatment, while saline-treated mice gained weight (Figure 3F), suggesting that reduced food intake may contribute to the decrease in blood glucose observed. Consistent with this, untreated F-IRKO and F-IR/IGFRKO mice were severely hyperphagic, with a food intake double that of controls (Figure 3G) and concomitant polydipsia (Figure 3H).

**F-IRKO and F-IR/IGFRKO mice display beta cell hyperplasia**

Elevated insulin levels were associated with massive pancreatic islet hyperplasia, which was easily detected by 3 months of age in F-IRKO and F-IR/IGFRKO mice (Figure 4A and B). This correlated with increased beta-cell proliferation as illustrated by the ~4-fold increase in Ki67 labeling (Figure 4C and D). No changes in insulin levels or pancreatic islet size were observed in F-IGFRKO mice. Expression of Angptl8/betatrophin (also known as RIFL or lipasin (22; 23)) was increased by 1.8-fold in livers of 2.5 week old lipodystrophic mice but returned to normal by 3 months of age. Expression of SerpinB1, a recently identified beta cell growth factor (24), was significantly increased by ~2 fold in livers from 3 month old F-IRKO and F-IR/IGFRKO
mice (Figure 4E). Expression of SerpinB1 and Angptl8/betatrophin was not changed in WAT and BAT in 3 month-old mice (Suppl. Fig. 1).

**F-IRKO and F-IR/IGFRKO mice display ectopic lipid accumulation**

To determine if the lipodystrophic phenotype of F-IRKO and F-IR/IGFRKO mice was associated with ectopic lipid accumulation, we measured triglyceride levels in liver, muscle and heart from control and knockout mice. Indeed, triglycerides were elevated in both liver (25) and muscle and heart of lipodystrophic mice. Tibialis anterior (TA) muscle from 12-week-old F-IRKO and F-IR/IGFRKO mice showed ~2.5 fold increase in intracellular TG compared to control and F-IGFRKO muscles (Figure 5A). Nile red staining of muscle cross-sections confirmed this increase and indicated that the mitochondria-rich oxidative fibers specifically displayed the accumulation of lipid as evidenced by co-localization of Nile Red staining with the SDH-positive fibers (Figure 5B). There was no difference in percent of oxidative fibers in the knockouts compared to controls (Figure 5C). Expression of the glucose transporter Glut4 was decreased in F-IRKO and F-IR/IGFRKO muscle (Suppl. Fig. 2A and B), which could further exacerbate insulin resistance and glucose intolerance (26).

Interestingly, TA muscle weights were smaller in F-IRKO and F-IR/IGFRKO mice (Figure 5D) and muscle fiber size was significantly decreased compared to controls (Figure 5E). Quantitation of the cross-sectional area showed that F-IRKO and F-IR/IGFRKO muscles had higher percentage of small fibers ranging in size from 1000 to 2000 \( \mu m^2 \), compared to the normal distribution of muscle fiber size in control and F-IGFRKO mice (Figure 5F). The muscle atrophy in these lipodystrophic mice was not
explained by chronic upregulation of E3-ubiquitin ligases specific for muscle atrophy Atrogin-1 and MuRF-1, which were unchanged or decreased in F-IRKO and F-IR/IGFRKO mice (Suppl. Fig. 2C). Ectopic lipid accumulation in muscle is often associated with insulin resistance (27), but usually this occurs without muscle atrophy. We recently demonstrated that loss of IR in muscle causes significant muscle atrophy (15), and it is well known that hyperinsulinemia can induce IR degradation (28; 29). In F-IRKO and F-IR/IGFRKO muscle, IR protein was decreased by ~90% compared to controls, and this occurred with no change in IR mRNA levels (Figure 5G- I). In contrast to skeletal muscle, heart weight tended to be increased in 3 month old lipodystrophic mice, although this did not reach significance. Triglyceride content of the hearts, however, was already elevated in F-IRKO and F-IR/IGFRKO mice, similar to the changes observed in skeletal muscle (Suppl. Fig. 3A). This occurred with no difference in blood pressure between control and F-IR/IGFRKO mice (Suppl. Fig.4). At 52 weeks of age heart size and weight of F-IRKO and F-IR/IGFRKO mice was significantly higher compared to the controls, however, heart triglyceride content was not different among the groups, mirroring normalization of triglyceride accumulation in the skeletal muscle at this age (Suppl. Fig 3B,C). Taken together, these data demonstrate that the lipodystrophy in F-IRKO and F-IR/IGFRKO mice is associated with lipid accumulation in muscle and heart, and muscle atrophy, probably secondary to downregulation of IR by the state of chronic hyperinsulinemia.

Hyperglycemia and hyperlipidemia develop early but improve with age despite maintained lipodystrophy
The natural history of the disease progression in F-IRKO and F-IR/IGFRKO mice is illustrated in Figure 6, and documents sustained lipodystrophy throughout life. Subcutaneous/inguinal WAT, which appears first and was detectable in control mice even before weaning at 2.5 weeks of age, was already decreased by 50% in F-IR/IGFRKO mice at this time. From 5 to 52 weeks of age, subcutaneous WAT mass continually increased in controls, but remained decreased by >90% in F-IRKO mice and was hardly detectable in F-IR/IGFRKO mice at all ages (Figure 6A). Likewise, visceral/perigonadal WAT, which was barely detectable at 2.5 weeks and gradually increased with age in controls, was reduced by >90% in F-IRKO mice and was absent in F-IR/IGFRKO mice at all ages (Figure 6B). Consistent with the low white fat mass, circulating leptin and resistin levels remained low even at one year of age (Suppl. Fig.5).

In contrast to WAT, BAT undergoes significant development during embryogenesis and was present in similar amounts in 2.5 week-old control, F-IGFRKO and F-IRKO mice, but was undetectable in F-IR/IGFRKO mice (Figure 6C and Suppl. Fig.6). In the latter, BAT weight remained less than 90% of control levels at 5 and 12 weeks of age and was undetectable in one year-old animals. As noted above, surprisingly, in 5 week-old F-IRKO mice BAT was two-times heavier than controls due to increased lipid accumulation, and BAT mass remained elevated up to one year of age in these mice. In F-IGFRKO mice, BAT mass was reduced by ~25% at 5 to 52 weeks-of-age, but was morphologically identical to controls (Figure 6C and Suppl. Fig.6).

Metabolic abnormalities were present in F-IRKO and F-IR/IGFRKO mice as early as they could be measured. Fed glucose levels were already significantly elevated in F-IRKO and F-IR/IGFRKO mice by 2.5 weeks of age (226±34 and 231±43 mg/dl vs.
145±7 mg/dl in controls) and reached values of >500 mg/dl by 5 weeks of age. By 1 year of age, blood glucose levels unexpectedly improved (Figure 6D). Circulating TG and FFA levels, which were elevated throughout the early months of life in F-IRKO and F-IR/IGFRKO mice, also returned towards normal by one year of age (Figure 6E and F), whereas serum cholesterol levels remained elevated (Suppl. Fig.7). The ectopic lipid deposition in skeletal muscle and heart, and the decrease in muscle size observed in 3 month old F-IRKO and F-IR/IGFRKO mice also normalized in 1 year old mice, likely reflecting the changes in circulating lipid levels (Figure 6G and Suppl. Fig.3B and 8). However, by 1 year of age both F-IRKO and F-IR/IGFRKO lipodystrophic mice developed cardiac hypertrophy with heart weights double that of control mice (Suppl. Fig.4B).

Despite improved hyperglycemia, insulin levels which were elevated by 10- to 15-fold as early as 2.5 weeks in F-IRKO and F-IR/IGFRKO mice, remained elevated throughout life (Figure 6H). Likewise at 1 year of age F-IRKO and F-IR/IGFRKO mice remained hyperphagic, while their water intake dramatically reduced, reflecting improvement in blood glucose (Suppl. Fig.9). Beta-cell mass also continued to be highly increased in 1 year old lipodystrophic mice, indicating persistence of insulin resistance (Figure 6I and Suppl. Fig.10). This massive islet hyperplasia occurred without increase in the expression of either putative beta-cell growth factor, SerpinB1 and Angptl8/betatrophin, in liver at this age (Figure 6J).
DISCUSSION

In the present study we show that both insulin and IGF-1 signaling are essential for development and function of adipose tissue, although these two receptors play different roles in white and brown fat. In WAT, IR is more dominant in both development and maintenance of normal mass and gene expression. The IGF1R also plays a role in gene expression in WAT, as well as in maintenance of normal serum leptin and adiponectin levels, but has little effect on tissue mass. Despite the modest role of the IGFR1R alone, combined knockout of IR and IGF1R produces a more severe phenotype than knockout of IR alone, with greater reductions in fat mass and greater decreases in the expression of adipocyte markers, adipokines, and lipogenic enzymes, indicating an interaction between these two complementary pathways. In BAT, on the other hand, absence of IGFR produces only a small decrease in mass and no decrease in function, as assessed by ability to maintain body temperature in the cold. By contrast, loss of IR alone produces an increase in BAT mass due to increased triglyceride storage, but significant BAT dysfunction with inability to maintain body temperature in the cold. Again, IR/IGF1R double knockout produces a profound decrease in BAT mass and profound cold intolerance, indicating a strong interaction between IR and IGF1R in BAT formation and function.

The striking difference and ultimate interaction between IR and IGF1R knockouts likely stems, at least in part, from differential expression of these two receptors during adipocyte development. The IGF1R is highly expressed in undifferentiated preadipocytes, while IR is low at this stage, whereas in mature adipocytes IR increases to high levels, dominating over IGF1R (3; 5). Adiponectin expression is also the highest in
mature adipocytes (30), and low in preadipocytes (31; 32), leading to progressive gene recombination through the differentiation process. IGF1R may be deleted in the developing adipose tissues at a stage when expression of the IR with its overlapping pathways is high enough to rescue the absence of IGF1R, resulting in a minimal phenotype. Conversely, IR is probably deleted in differentiating adipocytes, and in the presence of low IGF1R levels, producing a more severe phenotype. Lineage tracing studies using the adiponectin promoter indicate that adipocyte commitment in subcutaneous adipose tissue takes place during E14-E18, but in visceral adipose tissue occurs gradually during the post-natal period (33). The somewhat stronger phenotype in subcutaneous tissue may be due to the more sudden loss of insulin signaling in this depot, while a gradual decrease in visceral tissue may produce a milder phenotype.

We and others have shown that “fat-specific” knockout of genes using Cre-recombinase expressed under the aP2-promoter versus the adiponectin-promoter can lead to different phenotypes. This is, in part, due to the higher expression and recombination efficiency of adiponectin-driven Cre and to the greater specificity of its expression in fat (10; 11; 13). In this study, it was impossible to directly measure recombination efficiency since the lipodystrophic mice have a profound loss of adipose tissue combined with infiltration by inflammatory cells and fibroblasts, resulting in a change in tissue composition that would obscure accurate gene recombination results.

Interestingly, deletion of IR and IGF1R using aP2-Cre versus Adipo-Cre produces different phenotypes. aP2-Cre F-IGFRKO mice have an increase in WAT mass and increased overall growth associated with a modest increase in circulating IGF-1 levels (8), whereas Adipo-Cre F-IGFRKO mice have modest reductions in both WAT and BAT
mass but are of normal size, despite a 73% increase in circulating IGF-1 levels (Suppl. Fig.11). Likewise, mice with knockout of the IR or both the IR and IGF1R created using the aP2-Cre have only a moderate reduction in WAT and exhibit improved glucose tolerance when challenged with high fat diet (6; 9), whereas Adipo-Cre F-IRKO and F-IR/IGFRKO mice have almost no WAT and are overtly diabetic. This is similar to mice with Pparγ deleted using aP2-Cre, which display modest fat reduction and normal glucose levels (34; 35), whereas mice with Pparγ deletion driven by Adipo-Cre exhibit almost a complete loss of WAT and BAT, marked hepatic steatosis and severe diabetes (36).

The lipodystrophic syndrome observed in F-IRKO and F-IR/IGFRKO mice is similar to metabolic derangement of human generalized lipodystrophy including low leptin levels, marked insulin resistance, hyperlipidemia and fatty liver disease (21; 37). Leptin replacement reduces blood glucose levels and hepatic steatosis in human patients with lipodystrophy (38), and leptin replacement also normalizes blood glucose levels in F-IRKO and F-IR/IGFRKO mice. The effects of leptin are, at least in part, secondary to reduced food intake, and can be mimicked by fasting alone (25).

The lipodystrophic phenotype present in the knockout mice is associated with insulin resistance and dramatically elevated insulin levels throughout life. This is due to massive beta-cell hyperplasia, indeed the largest level of beta-cell observed in any of our tissue specific knockout mice. Angptl8/betatrophin (22; 23) and SerpinB1 (24) have recently been shown to be beta-cell growth factors that contribute to the islet hyperplasia in insulin resistant states. In our models of lipodystrophy, expression of Angptl8 and SerpinB1 in liver is modestly increased when the mice are young and may contribute to
the increased beta cell proliferation. On the other hand, at one year of age, F-IRKO and F-IR/IGFRKO mice continue to have massive β cell hyperplasia despite normal expression levels of these putative beta-cell growth factors, suggesting the possibility of other mechanisms driving beta-cell growth in these lipodystrophic, insulin resistant states.

Due to the inability to store fat in adipose tissues, lipodystrophic F-IRKO and F-IR/IGFRKO mice display ectopic lipid accumulation in liver and skeletal muscle. In muscle, this is associated with decreased fiber size and overall reduced skeletal muscle mass. This reduced muscle mass could reflect the increased muscle breakdown due to the uncontrolled diabetes in these mice or may be due to the marked down regulation of IR levels in skeletal muscle or some combination of these events (15). Muscle fiber size and intramyocellular TG normalized in older mice, as did many of the metabolic parameters including circulating glucose, fatty acid and triglycerides levels. On the other hand, adipose tissue mass remained dramatically reduced and insulin levels remained elevated. The improvement in glucose levels is likely due to the worsening of fatty liver disease, which progressed to NASH and development of dysplastic hepatic nodules with increased glycolysis and reduced gluconeogenesis. This is described in detail in the following article (25). Whether this accounts for reversal of the other metabolic abnormalities remains to be determined.

In summary, insulin and IGF-1 signaling play critical and distinct roles in the development and function of white and brown fat. Insulin receptor is most essential for formation and maintenance of WAT mass. IR is dispensable for development and/or maintenance of BAT mass, as long as IGF1R is present, but is essential for normal BAT-
dependent thermogenesis. Lack of both IR and IGF1R in fat leads to severe generalized lipodystrophy of both WAT and BAT accompanied by diabetes, insulin resistance with increased beta cell mass, and ectopic lipid accumulation. The hyperglycemia and hyperlipidemia of this lipodystrophy disappear over time without recovery of lipodystrophy or reversal of insulin resistance. This model demonstrates the unique pathogenesis of the different features of the metabolic syndrome associated with lipodystrophy.
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J.B. initiated the project, generated the data and wrote the manuscript. S.S. generated the data and wrote the manuscript. A.E., A.K, M.T.K., R.N.K and B.T.O. generated the data and reviewed the manuscript. C.R.K. oversaw the project, contributed to discussion, and helped write the manuscript. C.R.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. There are no potential conflicts of interest relevant to this article by any of the authors.
References


FIGURE LEGENDS

Figure 1: Lipodystrophy in mice lacking IR and IR/IGF1R in fat
A) Subcutaneous (inguinal) and visceral (epididymal) white adipose tissue weights in 3 month old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 12 to 30 animals per group. B) H&E stained sections of subcutaneous and visceral adipose tissues from the same mice in panel A. C) Circulating leptin, adiponectin and resistin levels in 3 month old random-fed mice. Results are mean ± SEM of 7 to 17 animals per group. D) mRNA levels in visceral white adipose tissue from 3 month old control, F-IGFRKO and F-IRKO mice. Results are mean ± SEM of 6 to 10 animals per group. * indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001). # indicates a significant difference between F-IRKO and F-IR/IGFRKO mice, p<0.05.

Figure 2: Importance of IR and IGF1R in BAT development and function.
A) BAT weight in 3 month old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 12 to 30 animals per group. B) H&E stained sections of BAT from the same mice in panel A. C-E) BAT mRNA levels from 3 month old mice. Results are mean ± SEM of 6 to 10 animals per group. F) Rectal temperature measured in 3 month old mice every 30 min for 3 hours during exposure to a 6°C environment. Results are mean ± SEM of 10 to 15 mice per group. G) mRNA levels in BAT from 3 month old mice at room temperature or exposed to a 6°C environment for 3h prior to sacrifice. Results are mean ± SEM of 5-6 mice per group. H) Mitochondrial/Nuclear DNA ratio (n=9-11) and basal oxygen consumption rate (n=4-6) in BAT from 3 month old control
and F-IRKO mice. Results are mean ± SEM. *indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001).

Figure 3: F-IRKO and F-IR/IGFRKO mice display lipoatrophic diabetes which is reversible with leptin treatment.
A) Blood glucose and B) serum insulin levels in random-fed 3 month old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 7 to 17 animals per group. C) Insulin tolerance test was performed in 3 month old mice as described in Methods. Results are mean ± SEM of 12 animals per group. D) Serum triglycerides, free fatty acids and cholesterol levels in random-fed mice at 3 months of age. Results are mean ± SEM of 7 to 17 animals per group. E) Blood glucose change and F) body weight change in random-fed 3 month old F-IRKO and F-IR/IGFRKO mice during 2 weeks of leptin (10 µg/mouse/day) or saline treatment using Alzet osmotic minipumps. Saline-treated F-IRKO and F-IR/IGFRKO mice were pooled into a single control group. Results are mean ± SEM of 6 mice per group. G) Food intake and H) water intake were measured in metabolic cages in 3 month old control, F-IRKO and F-IR/IGFRKO mice. Results are mean ± SEM of 6 to 10 mice per group. (*, p<0.05; **, p<0.01; ***, p<0.001).

Figure 4: Beta cell hyperplasia in F-IRKO and F-IR/IGFRKO mice
A) H&E stained pancreatic sections from control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at 3 months of age. B) Percent islet cell area as compared to total area of the pancreas. Results are mean ± SEM of 4-5 animals per group. C) Immunofluorescence staining for insulin, Ki67 and DAPI in pancreatic sections of 3
month old mice. D) Percent of Ki67 positive beta cells. Results are mean ± SEM of 4-5 animals per group. E) Angptl8/Betatrophin and SerpinB1 mRNA levels in livers from 2.5 and 12 week old mice. Results are mean ± SEM of 5 to 8 animals per group.

*indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001).

Figure 5: Ectopic lipid accumulation and decreased muscle size in F-IRKO and F-IR/IGFRKO mice

A) Triglyceride content in tibialis anterior (TA) muscles from 3 month old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. B) Nile red staining shows lipid accumulation co-localizes with oxidative SDH-positive (purple) stained myofibers in TA from F-IRKO and F-IR/IGFRKO (bar = 100 µm). C) Percent of SDH-positive oxidative (purple) fibers per high power field (HPF). D) Weight of TA muscle from control and knockout mice at 3 months of age. Results are mean ± SEM of 12 to 30 animals per group. E) Laminin immunofluorescence and F) cross-sectional area distribution of laminin-stained fibers in TA muscles from 3 month old control and knockout mice (bar = 200 µm). Results are mean ± SEM of 3 animals per group. G) Western blot and H) densitometric quantification of protein levels of insulin receptor (IR) in quadriceps muscle of 3 month old mice. I) mRNA levels of IR in quadriceps muscle from control and knockout mice. Results are mean ± SEM of 3 to 6 animals per group.*indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001).

Figure 6: Disease progression in mice with IR and/or IGF1R knockout in fat
A) Subcutaneous (inguinal) WAT, B) visceral (epididymal) WAT, and C) interscapular BAT mass from mice at 2.5, 12 and 52 weeks of age. Results are mean ± SEM of 7 to 17 animals per group. D) Blood glucose was measured in random-fed control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at the indicated ages from 2.5 week old to 1 year old. Results are mean ± SEM of 8 to 15 animals per group. E) Serum triglycerides and F) serum free fatty acids in random-fed mice at 2.5, 12 and 52 weeks of age. Results are mean ± SEM of 7 to 17 animals per group. G) Triglyceride content of tibialis anterior (TA) muscles from 52-week-old control and knock out mice. Results are mean ± SEM of 5 to 8 animals per group. H) Serum insulin levels were measured in random-fed control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at the indicated ages from 2.5 week old to 1 year old. Results are mean ± SEM of 8 to 15 animals per group. I) H&E stained pancreatic sections from 1 year old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. J) Angptl8/Betatrophin and SerpinB1 mRNA levels in livers from 52 week old mice. Results are mean ± SEM of 5 to 8 animals per group.
**Figure 2**

A. Rectal temperature (°C)

![Graph showing rectal temperature](image)

- Control
- F-IRKO
- F-IGFRKO
- F-IR/IGFRKO

B. Microscopic images of Control vs. F-IGFRKO

Control vs. F-IGFRKO images demonstrate differences in tissue structures.

C. mRNA (fold change)

- Leptin
- Hoxc8
- Zic1
- LHX8
- Tbx1

![Graph showing mRNA levels](image)

D. mRNA (fold change)

- UCP1
- PRDM16
- Elovl3
- Cidea
- Dio2
- Adrb3
- Tfam

![Graph showing mRNA levels](image)

E. mRNA (fold change)

- C/EBPα
- PPARγ
- aP2
- GLUT4
- FAS
- HSL
- ATGL

![Graph showing mRNA levels](image)

F. Rectal temperature (°C)

![Graph showing rectal temperature changes](image)

- Control
- F-IRKO
- F-IGFRKO
- F-IR/IGFRKO

G. mRNA (fold change)

- UCP1
- PGC1α
- Elovl3
- Cidea
- Tfam
- Adrb3

![Graph showing mRNA levels](image)

H. OCR (AUC)

- Control
- F-IRKO

![Graph showing OCR levels](image)
**Figure 4**

**A.**

Comparison of control and F-IGFRKO mice. Images show liver sections stained with hematoxylin and eosin. The sections highlight differences in cellular structure and distribution between the two groups.

**B.**

Graph showing the percentage of β-cell area.

**C.**

Immunofluorescence images of insulin, Ki67, and DAPI staining. The images capture the distribution and expression of these markers in control and F-IGFRKO mice.

**D.**

Graph showing the percentage of Ki67+ cells.

**E.**

Bar graph comparing Angptl8/Betatrophin and SerpinB1 mRNA levels in 2.5 week and 12 week old mice across different genotypes.

Diabetes
Figure 5

A. Muscle Triglycerides

B. Nile Red

C. Oxidative Fibers

D. TA muscle weight (mg)

E. Laminin

F. Cross Sectional Area (µm²)

G. IR Protein

H. IR mRNA
Figure 6

A. Subcutaneous WAT (g)

B. Visceral WAT (g)

C. BAT (g)

D. Blood glucose (mg/dl)

E. Serum triglycerides (mg/dl)

F. Serum Free Fatty Acids (mEq/L)

G. Muscle Triglycerides (mg/g tissue)

H. Muscle Angptl8/Betatrophin mRNA

I. Muscle Serpin B1 mRNA

J. Angptl8/Betatrophin mRNA

Legend:
- **: p < 0.01
- *: p < 0.05
- ***: p < 0.001

Control  F-IR/IGFRKO  F-IRKO  F-IGFRKO

Diabetes

Insulin (ng/ml)
Supplemental Figure 1: Betatrophin and Serpin B1 mRNA levels in white adipose tissue (epididymal) and interscapular brown adipose tissue from 3 month old control and F-IRKO mice. Results are mean ± SEM of 6 to 10 mice per group.
Supplemental Figure 2: A) Q-PCR and (B) Western blot of Glut4 in quadriceps muscle from 12-week-old control and knockout mice. (C) Q-PCR of E3-ubiquitin ligases in control and knockout muscle. (*-p<0.05, **-p<0.01, ***-p<0.001 vs. control)
Supplemental Figure 3: A) Heart weight and heart triglyceride content of 3 month old control and knockout mice. B) Heart weight and heart triglyceride content of 1 year old control and knockout mice. Results are mean ± SEM of 12 to 24 mice per group for heart weight and 6-7 mice per group for TG content. (*, p<0.05; **, p<0.01; ***, p<0.001). C) Image of whole heats and H&E sections of hearts from control and knockout mice at 52 weeks of age.
Supplemental Figure 4: A) Blood pressure measured by the tail-cuff method and B) Heart rate of 3 month old control and F-IR/IGFRKO mice. Results are mean ± SEM of 8 mice per group.
**Supplemental Figure 5:** Circulating leptin and resistin levels in random fed control, F-IRKO and F-IR/IGFRKO mice with age. Results are mean +/- SEM of 5-10 animals per group.
Supplemental Figure 6: H&E stained sections of BAT from control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice at the indicated times from 2.5 to 52 weeks of age (one representative section from 5 mice per group is shown)
Supplemental Figure 7: cholesterol levels in random-fed mice at 2.5, 12 and 52 weeks of age. Results are mean ± SEM of 7 to 17 animals per group. (*-p<0.05, **-p<0.01, ***-p<0.001 vs. control)
Supplemental Figure 8: A) Muscle weight of tibialis anterior (TA) from control and knockout mice. B) Nile Red staining in TA muscle from control and knockout mice. *indicates a significant difference compared to controls (*, p<0.05; **, p<0.01; ***, p<0.001).
Supplemental Figure 9: Food and water intake in 3 month old (data from Figure 3 G and H shown as comparison) and 1 year old control, F-IRKO and F-IR/IGFRKO mice. Results are average ±SEM of 5 to 11 mice per group.
**Supplemental Figure 10:** H&E stained sections of pancreas from 1 year old control, F-IGFRKO, F-IRKO and F-IR/IGFRKO mice. Three representative sections from 5 mice per group are shown.
Supplemental Figure 11: serum IGF-1 levels in 3 month old random fed control, F-IGFRKO, F-IRKO, and F-IR/IGFRKO mice (***p<0.001 vs. control)