Role of Insulin Action and Cell Size on Protein Expression Patterns in Adipocytes

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Abstract

Mice with a fat-specific insulin receptor knockout (FIRKO) exhibit a polarization of white adipose tissue into two populations of cells - one small (diameter < 50 µm) and one large (diameter > 100 µm) – accompanied by changes in insulin-stimulated glucose uptake, triglyceride synthesis and lipolysis. To characterize these subclasses of adipocytes, we have used a proteomics approach in which isolated adipocytes from FIRKO and control (IR lox/lox) mice were separated by size, fractionated into cytosolic and membrane subfractions and analyzed by sucrose gradient, SDS-PAGE and mass spectrometry. A total of 27 alterations in protein expression at key steps in lipid and energy metabolism could be defined that were coordinately regulated by adipocyte cell size, impaired insulin signaling or both. Nine proteins, including vimentin, EH-domain containing protein 2, elongation factor 2, glucose regulated protein 78 (GRP 78), transketolase, succinyl CoA transferase were primarily affected by presence or absence of insulin signaling, while 21 proteins, including myosin non-muscle form A, annexin 2, annexin A6, and 47 kD heat shock protein, were regulated in relation to adipocyte size. Of these 27 alterations in protein expression, 14 changes correlated with altered levels of mRNA, while the remaining 13 were due to changes in protein translation or turnover. These data suggest an intrinsic heterogeneity in adipocytes with differences in protein expression patterns due to transcriptional and post-transcriptional alterations related to insulin action and cellular lipid accumulation.
Introduction

Adipose tissue plays a central role in the pathogenesis of diabetes and obesity. White adipose tissue provides the primary site of energy storage in the body and also serves as an important endocrine cell through secretion of hormones such as leptin, adiponectin (ACRP 30), TNFα, resistin and other cytokines (1). Brown adipose tissue is the major site of energy expenditure through expression of uncoupling protein 1 and its role in thermogenesis (2).

Insulin signaling plays important role in lipid storage and the process of adipogenesis for both white and brown adipocytes. The loss of insulin action selectively in adipose tissue in mice with a fat-specific insulin receptor knockout (FIRKO) leads to profound changes in adipocyte function, including changes in glucose metabolism, lipid metabolism and protein expression (3). FIRKO mice have reduced fat mass and are protected against age- and diet-related obesity and its associated metabolic abnormalities, including glucose intolerance. In addition, these mice have increased longevity, despite normal or increased food intake (4). Adipose tissue-specific insulin receptor knockout in FIRKO mice also causes heterogeneity of white adipose tissue with polarization into small (diameter < 50 µm) and large (diameter > 150 µm) subclasses of adipocytes (3). Western blot analysis of candidate molecules reveals changes in the expression of several key adipocyte proteins, such as ACRP 30, fatty acid synthase (FAS), SREBP-1, C/EBPα, and GLUT1 glucose transporter (3), suggesting that knockout of the insulin receptor unmasks a intrinsic heterogeneity of cells in fat tissue. Analysis of gene expression using oligonucleotide microarrays has demonstrated that this is accompanied by changes in mRNA levels of 111 known cDNAs, as well as many unknown ESTS, some of which are
related in insulin signaling alterations and others to differences in adipocyte size (5). The recent development of unbiased proteomic analysis using gel electrophoresis and other separation techniques coupled with mass spectrometry, however, now allows the characterization of thousands of proteins from complex samples to reveal previously unrecognized connections between biochemical processes and protein expression patterns (6).

In the present study, we have used a multidimensional proteomics approach to test the hypothesis that insulin receptor knockout in FIRKO mice unmasks a naturally occurring heterogeneity of adipocytes that results in differential lipid storage resulting in subsets of small and large adipocytes. By comparing these findings to mRNA expression we can define unique and complementary levels of protein and mRNA regulation (7).
Materials and Methods

Animals

IR (lox/lox) mice were derived as previously described (8) and maintained on a mixed (C57Bl/6 X 129/Sv) genetic background. Adipose tissue or fat specific insulin receptor knockout mice (FIRKO) were derived by crossing IR lox/ox mice with mice that were heterozygous for the IR and were heterozygous of a transgene expressing the Cre recombinase under the control of the fatty acid binding protein aP2 promoter/enhancer [aP2-Cre-IR(lox/+)] (3). Animals were housed in virus-free facilities on a 12 hr light/dark cycle (0700 on- 1900 off) and were fed a standard rodent chow (Mouse Diet 9F, PMI Nutrition International) and allowed water ad libitum. All protocols for animal use and euthanasia were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with NIH guidelines. Genotyping was performed by PCR using genomic DNA isolated from the tail tip as previously described (3).

Histology

Tissues were fixed in 10% buffered formalin and imbedded in paraffin. Multiple sections (separated by 70-80 µm each) were obtained from gonadal fat pads and analyzed systematically with respect to adipocyte size and number. Staining of the sections was performed with hematoxylin/eosin. For each genotype and gender at least 10 fields (representing approximately 100 adipocytes) per slide were analyzed. Images were acquired using BX60 microscope (Olympus, NY) and a HV-C20 TV camera (Hitachi, Japan) and were analyzed using Image-Pro Plus 4.0 software.
Isolation of adipocytes and separation into small and large adipocytes

Animals were sacrificed, and epididymal fat pads were removed. Adipocytes were isolated by collagenase (1 mg/ml) digestion. Separation of cells into small and large adipocytes was achieved by filtering the adipocyte suspension through a 75 µm pore size nylon mesh screen (Figure 1). Aliquots of adipocytes were fixed with osmic acid and counted in a Coulter counter (9). Adipocyte size was determined by dividing the lipid content of the cell suspension by the cell number (9).

Cell fractionation

Isolated small and large adipocytes were layered with 5 ml dinonylphthalate and centrifuged for 5 min at 500 rpm in Beckman GPKR centrifuge. Adipocytes were removed and washed twice, once in DMEM and once in cytosol buffer (cytosol buffer: 25 mM Hepes, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 0.2 M sucrose, 1 mM ATP, 5 mM creatine phosphate, 1 mM dithiothreitol, 1 mM benzamidine, 4 µg/mL leupeptin, 0.01 mg/ml tosyl-L-arginine methyl ester, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM phenanthrine). Buffer was removed and fresh cytosol buffer added to the cells at a volume half of the cell volume. Adipocytes were homogenized by 10 passes at 500 rpm using a motor-driven Teflon Pestle Homogenizer (Schuett, Germany). After 5 min spin at 500 x g, the postnuclear supernatant was centrifuged at 72,000 rpm in a TLA 100.3 rotor (Beckman) for 15 min at 4°C. The supernatant was removed and saved as the cytosol fraction. The postnuclear pellet was resuspended in 100 µl of cytosol buffer and incubated with 2.5 M KCl to a final concentration of 0.5 M KCl on ice for 10 min. The suspension was centrifuged at 72,000 rpm in a TLA 100.3 rotor (Beckman) for 15 min at 4°C. The supernatant was removed as
the KCl extract. The pellet was solubilized in cytosol buffer supplemented with CHAPS to a final concentration of 6 mM, vortexed, and cytosol buffer added to bring the final concentration of CHAPS to 3 mM. The suspension was vortexed continuously for 10 min, and protein determination performed using the Bradford assay (Bio-Rad). Equal concentrations of protein were layered on top of a 1.8 ml 10-30% sucrose gradient. The sucrose gradients were centrifuged at 35,000 rpm in a TLS55 rotor (Beckman) for 20 hr at 4°C. Each gradient was fractionated into ten 200 µl fractions from the top and then analyzed on 7.5-15% SDS-PAGE gels. Gels were stained with Sypro Ruby protein stain (Molecular Probes) and photographed with a charge-coupled device camera after illumination with a 302 nm wavelength Ultra-Lum electronic UV transilluminator.

Mass Spectrometry

Proteins bands were excised from the polyacrylamide gel and digested in-gel with trypsin (0.1 mg/ml trypsin (sequence grade, Promega) in 25 mM NH₄HCO₃, pH 8 for 12-16 h at 37 °C). The hydrolyzates were then analyzed by either electrospray ion trap mass spectrometry (ESI) or matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). To analyze by ESI ion trap mass spectrometry, the digests were run out on a LC Packings Ultimate nano HPLC with a 100 micron C18PM column in a solvent A (0.1% formic acid, 3.5% acetonitrile) and solvent B (0.1% formic acid in 70/30 acetonitrile/water). Peptides were eluted with a linear gradient from 100% solvent A to 60% solvent B in 40 min at a flow of 500 nanoliters/min. Peptides were eluted directly into the LCQ Deca ESI ion-trap (LC/MS) mass spectrometer equipped with data-dependent acquisition and a high resolution scan performed. A higher energy MS/MS
scan was performed following the initial scan to verify peptide identifications. Peptides were searched using the Sequest software developed by John Yates and Jimmy Eng (University of Washington). To analyze by MALDI-TOF, the digested samples were further concentrated and desalted with Millipore Zip Tip C18 microtips. Peptide masses were determined using a Kratos Analytical Axima CFR MALDI-TOF spectrometer equipped with a curved field reflectron. Peptide masses were searched against the non-redundant protein data base using MS-Fit of the Protein Prospector program, a program available from the World Wide Web site of the Mass Spectrometry Facility of the University of California San Francisco. Fragmentation data from individual peptides via post source decay analysis was searched against the non-redundant protein database using the Protein Prospector program MS-Tag.

**Immunoprecipitation and Western Blot Analysis**

Immunoprecipitations and Western blot analyses were performed on homogenates from isolated small and large adipocytes. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, which were blocked with 1% bovine serum albumin in Tris-buffered saline containing 20% Tween, and incubated with the indicated primary antibodies. At least three blots of samples from four (controls) to eight animals (FIRKO) of each genotype were scanned using a Molecular Dynamics Storm PhosphoImager and quantified using ImageQuant version 4.0 software. All values are expressed as mean ± SEM unless otherwise indicated. Statistical analyses were carried out using two-tailed Student’s unpaired t-test and between more than two groups by analysis of variance (ANOVA). Significance was rejected at p ≥ 0.05.
Microarray Analysis of mRNA Levels

This work is described in detail in the accompanying paper (5). In brief, total RNA was isolated from isolated pooled small and large adipocytes from at least 8 FIRKO mice and at least 4 IR lox/lox mice. Double-stranded cDNA synthesis was reverse transcribed from 15 µg of isolated mRNA by using the SuperScript choice system (Invitrogen) in addition to using an oligo(dT) primer containing a T7 RNA polymerase promoter site. Double stranded cDNA was purified with Phase Lock Gel (Eppendorf). Biotin-labeled cRNA was transcribed by using a BioArray RNA transcript labeling kit (Enzo). A hybridization mixture containing 15 µg of biotinylated cRNA, adjusted for possible carryover of residual total RNA, was prepared and hybridized to mouse Affymetrix MG-U74A-v2 chips. The chips were washed, scanned, and analyzed with GENECHIP MAS V.4.0.

For each condition (FIRKO small and large, IR lox/lox small and large) 5 chips were analyzed. All chips were subjected to global scaling to a target intensity of 1,500 to take into account the inherent differences between the chips and their hybridization efficiencies. The background and the scaled noise of each of the chips were averaged.

The data analysis was performed as described by Yechoor et al. (10).
Results

Adipocyte Heterogeneity in FIRKO Mice

Adipose tissue specific insulin receptor knockout causes heterogeneity of white adipose tissue with a polarization into small (diameter < 50 µm) and large (diameter > 100 µm) subclasses of adipocytes. Histological sections representative of white adipose tissue in FIRKO and IR lox/lox mice demonstrate a relatively uniform and normally distributed adipocyte size in white adipose tissue from IR lox/lox mice, whereas the fat pads from FIRKO mice contain a mixed population of large and small adipocytes (Figure 1). We have previously shown that the polarization of adipocytes from FIRKO mice is accompanied by differences between small and large adipocytes in insulin-stimulated glucose uptake, glucose metabolism, triglyceride synthesis, and lipolysis, as well as in the expression of candidate proteins of adipocyte differentiation and metabolism (3). Insulin receptor expression in both large and small adipocytes of FIRKO mice was reduced by 85-99% as compared to the controls, indicating that the heterogeneity in adipocyte size in FIRKO mice was not due to differences in efficiency of insulin receptor gene recombination in small and large adipocytes. The three littermate control groups: WT, IR (lox/lox), and aP2-Cre mice were indistinguishable with regard to physiologic and metabolic parameters and possess the same mixed genetic background as the FIRKO mice, including C57Bl/6J, 129Sv, and FVB. Therefore, for all subsequent proteomics and genomics studies, IR lox/lox mice were used as controls.

Proteomic Analysis

To further characterize protein expression at the whole proteome level, adipocytes were isolated from epididymal fat pads from 3 months old male FIRKO and IR lox/lox
mice and separated into small (< 75 µm) and large (> 75 µm) by filtration through nylon mesh screen. Each population of cells was further fractionated into membrane and cytosol fractions, and protein from each was loaded onto 10-30% sucrose gradients, centrifuged for 20 h and then separate on 7-15% SDS-PAGE gels. Resolved proteins were visualized by Sypro Ruby protein stain, and differentially expressed proteins bands were excised from the gel, tryptically digested and analyzed by either sequencing ESI-mass spectrometry or peptide mass mapping fingerprinting (MALDI-TOF) as described in Methods. A total of three experiments were performed for each cell subfraction. Each experiment consisted of the comparison between FIRKO small and large, and IR lox/lox small and large adipocyte protein extracts in the KCl fraction of ionic bound membrane proteins, the CHAPS fraction of integrated membrane proteins and the cytosol fraction (Figure 1).

Comparing the protein expression profiles of small and large adipocytes from FIRKO and IR lox/lox mice, 27 differentially expressed proteins were identified (Table 1). For 14 of the proteins identified by proteomics, additional Western blot analysis was performed, and in each case this confirmed the protein expression pattern as detected by SDS-PAGE (Figures 2-4).

Analysis revealed a regulation of protein expression both as a function of adipocyte size, i.e. different protein expression patterns in large and small adipocytes independent from the insulin receptor knockout in adipocytes from FIRKO mice, as well as a function of impaired insulin signaling, i.e. in FIRKO adipocytes as compared to adipocytes from IR lox/lox mice independent of size. A coordinated regulation of protein levels with decreased protein expression in small adipocytes as compared to large
adipocytes, as well as decreased protein expression in adipocytes from FIRKO mice as compared to the controls could be identified for several proteins of lipid metabolism (fatty acid synthase, CPT 2, acyl CoA dehydrogenase, aP2, CD 36, methylmalonate-semialdehyde dehydrogenase), as well as for mitochondrial proteins (cytochrome C, succinyl CoA transferase, citrate synthase, HSP 60) (Table 1).

**Protein expression in function of the adipocyte size**

Comparison of the gradient fractions from small and large FIRKO and IR lox/lox adipocytes by SDS-PAGE revealed major differences between small and large adipocytes both from FIRKO and IR lox/lox mice (Figure 2). In three independent experiments analyzing “integral” membrane proteins (the CHAPS extract of membranes), myosin heavy chain, non-muscle form A, appeared only in large adipocytes from both FIRKO and IR lox/lox mice (Figure 2). This differential expression, with no detectable myosin heavy chain, non-muscle form A protein expression in small adipocytes and easily detectable levels in extracts of large adipocytes in both FIKRO and control mice was confirmed by Western blotting with a specific antibody (Figure 2). Within the limits of detection and this approach, this was the only protein exclusively detected in one cell type versus the other.

Significantly decreased protein expression in small as compared to large adipocytes from both FIRKO and IR lox/lox mice was detected for several other proteins, and could be categorized into three different patterns (Figure 3 A-C). The first pattern is characterized by decreased protein expression in small adipocytes of both FIRKO and IR lox/lox mice as compared to large adipocytes. This was the case for the expression of the key enzyme in fatty acid synthesis, the fatty acid synthase (FAS), carnitine palmitoyl
transferase 2 (CPT-2), the fatty acid binding protein aP2, clathrin heavy chain, carbonic anhydrase 3, and cyclophilin A (Figure 3A). The second pattern represented proteins that were decreased only in small adipocytes of FIRKO mice, but not changed in small adipocytes from IR lox/lox mice. This pattern included annexin A6, the heat shock protein HSP 47 and HSP 60, acyl CoA dehydrogenase, citrate synthase, acetyl CoA dehydrogenase, xanthine dehydrogenase, cytochrome C, and the glucose-regulated protein (GRP) 58 (Figure 3B). The third pattern of protein expression was characterized by proteins whose levels were decreased in small adipocytes of IR lox/lox mice, but normally expressed in small adipocytes of the FIRKO mouse. This pattern was observed for annexin II and pyruvate dehydrogenase (Figure 3C).

Thus, proteomic analysis demonstrated that key enzymes and other molecules involved in lipid and fatty acid metabolism are commonly decreased in small adipocytes as compared to large adipocytes. This could explain, at least in part, our observation that there are differences in triglyceride storage and triglyceride and fatty acid synthesis between large and small adipocytes (3).

Consequences of impaired insulin signaling on protein expression patterns

To further elucidate the effect of impaired insulin signaling on different protein expression patterns, we analyzed proteins that might be regulated in response to the insulin receptor knockout. This includes proteins with either decreased levels in both large and small FIRKO adipocytes (Figure 4A) or increased expression in both large and small FIRKO adipocytes (Figure 4B) as compared to their respective controls.

Decreased protein expression in both FIRKO large and small adipocytes was observed for the fatty acid translocase CD 36, the EH domain containing protein 2,
elongation factor 2, succinyl CoA transferase, and methylmalonate semialdehyde dehydrogenase (Figure 4A). Conversely, an expression pattern with increased protein levels in both large and small FIRKO adipocytes was identified for vimentin, the glucose regulated protein 78, aldehyde dehydrogenase 2, and transketolase (Figure 4B).

It is worth noting that the lack of insulin signaling due to knockout of the insulin receptor in FIRKO adipocytes caused alterations in proteins involved in both adipocyte differentiation and adipocyte metabolism. Differentially regulated proteins which could be associated with adipocyte differentiation process included proteins involved in cytoskeletal function (vimentin), protein processing (GRP 78) and protein synthesis (elongation factor 2). Other proteins with differential expression were related to fatty acid metabolism (CD 36, methylmalonate semialdehyde dehydrogenase), glycolysis (transketolase) and other metabolic pathways (aldehyde dehydrogenase 2, succinyl CoA transferase). Thus, the changes in protein expression pattern suggest that the phenotype of the adipose tissue in FIRKO mice might be the result of both changes in the adipocyte differentiation program and adipocyte metabolism in response to the insulin receptor knockout.

**Differentially regulated protein expression is at least in part confirmed by microarray analysis of gene expression patterns**

To investigate whether the observed changes in protein expression identified by proteomics were secondary to changes at the mRNA level or the result of post-transcriptional control, protein expression patterns were compared to mRNA levels determined by Affymetrix oligonucleotide microarrays. Some proteins, including non-muscle form A of myosin heavy chain, clathrin heavy chain, annexin A6, annexin II, EH...
domain-containing protein 2, citrate synthase, methylmalonate semialdehyde dehydrogenase, and GRP 58, were not present on the array, and therefore real-time PCR analysis was performed to obtain corresponding mRNA expression profiles for these proteins (Table 1). FAS, pyruvate dehydrogenase, xanthine dehydrogenase, acetyl CoA dehydrogenase, succinyl CoA dehydrogenase, aldehyde dehydrogenase 2, transketolase, GRP 78, vimentin, and CD 36 were all detectable on the microarray and in every case, the change in proteins was accompanied by a parallel change in mRNA expression (Table 1). However, the magnitude of the mRNA expression changes was in general lower than that in protein expression, suggesting that post-transcriptional modulation might contribute additionally to the expression changes observed by proteomics. The remaining 9 proteins (CPT 2, aP2, HSP 47, HSP 60, elongation factor 2, carbonic anhydrase 3, cyclophilin A, acyl CoA dehydrogenase, cytochrome C) were also represented on the array, however, no differences in the mRNA expression could be identified for these, indicating that the change in protein level was due primarily or exclusively to post-transcriptional events.
Discussion

Adipocytes play a central role in energy balance by serving as major sites of storage and expenditure and as endocrine cells, secreting adipokines and other molecules that regulate energy storage and metabolism in other tissues as well (11). We have recently shown that adipose tissue specific disruption of the insulin receptor in FIRKO mice has beneficial effects on the whole body glucose homeostasis and on longevity (3;4). FIRKO mice have a reduced total body fat mass and are protected against age-related obesity and its related metabolic abnormalities (3). Moreover, adipose tissue of FIRKO mice displays a heterogeneity of adipocyte size with polarization into small and large adipocytes that is accompanied by changes in lipid storage function, glucose metabolism and the expression of major modulators of adipocyte metabolism (FAS), secretory function (ACRP 30) and adipocyte differentiation (SREBP-1, C/EBPα) (3).

These alterations in the protein expression pattern could contribute to the heterogeneity of adipocytes and the beneficial effects of the insulin receptor knockout in adipose tissue on whole body glucose homeostasis. Heterogeneity in adipocyte cell size is also observed in mice with a deficiency of the enzyme hormone-sensitive lipase (HSL) (12;13) and mice with a genetic knockout of perilipin (14). Based on these and other observations, we have postulated that adipose specific disruption of the insulin receptor unmasks a naturally occurring heterogeneity of adipocytes with differential protein and gene expression patterns and different roles in lipid storage and other functions.

In this study we have used a multi-dimensional proteomics approach to detect differentially expressed proteins in isolated adipocyte samples that might play a role in the regulation of adipocyte biology and contribute to the heterogeneity of adipose tissue
in FIRKO and normal mice. We find that protein expression patterns are regulated in adipocytes, both as a function of cell size and as a function of impaired insulin signaling. These two categories of changes may define adipocyte subclasses and suggest that insulin resistance in adipocytes may cause profound changes in protein expression profiles. Furthermore, although only ~25% of adipocytes from control mice versus 50% of FIRKO adipocytes are smaller than 75 µm, the data of the present study show changes in protein expression in small adipocytes which in some cases are present in both genotypes, whereas in others affect adipocytes of only the knockout or control genotype. Thus, while small adipocytes could simply represent the natural precursors of large adipocytes (and some almost certainly do), the patterns of differential protein expression suggest that this is not strictly the case.

The only protein that was exclusively detected in large adipocytes of both genotypes in this study was the non-muscle myosin heavy chain, form A. Mutations of MYH9, the gene for the non-muscle myosin heavy chain causes the Fechtner syndrome (FTNS), a rare inherited condition characterized by progressive nephritis, macrothrombocytopenia, Dohle-like leukocyte inclusions, deafness, and cataract (15). Although the exact role of non-muscle myosin heavy chain in adipocyte biology is not known, this protein might be involved in the regulation of the integrity of cytoskeleton and cell shape (16), such that the lack of non-muscle myosin heavy chain could contribute to the smaller cell size. Recently, Bose et al. (17) have also suggested a role for non-muscle myosin, Myo1c, in glucose transport regulation. Myo1c binds to actin filaments, which are associated with actin-based tubulo-vesicular membranes containing GLUT4. These investigators also showed that decreased expression of endogenous
Myo1c inhibits insulin-stimulated glucose uptake via a phosphatidylinositol 3-kinase-independent insulin signaling pathway that controls the movement of intracellular GLUT4-containing vesicles to the plasma membrane (17). In the current study, reduced levels of the non-muscle type A form of myosin are found in the small adipocytes, suggesting that the absence of this form of myosin correlates with insulin sensitivity rather than insulin resistance. Clearly defining the role of each of these forms of non-muscle myosin in adipocytes will require further study.

We further demonstrate a coordinated down regulation of enzymes of β-oxidation (acyl CoA dehydrogenase, acetyl CoA dehydrogenase, CPT 2) and fatty acid synthesis (FAS) in small adipocytes as compared to large adipocytes. The common down regulation of these enzymes of fatty acid metabolism was confirmed by parallel mRNA expression changes as determined by the microarray analysis. These protein expression changes are in agreement with functional analysis of these same populations which shows decreased triglyceride storage in small adipocytes (3). Whether these protein expression changes are intrinsic to small adipocytes or secondary effects of an impaired substrate uptake needs to be further elucidated.

In contrast to our previous studies using whole cell lysates which demonstrated a no differences in the expression of aP2 protein in small adipocytes (3), the current study finds that this fatty acid binding protein is decreased in small adipocytes in both genotypes, but only in the membrane fraction. Thus, defining the specific compartmentalization of the protein adds another dimension to the proteomic analysis. Uysal, et al (18) have shown that genetically obese mice, which lack aP2 have improved lipid and glucose metabolism. Thus, decreased membrane associated aP2 protein in
small adipocytes might contribute to the improved lipid and glucose metabolism in these cells as compared to the population of larger adipocytes (3).

A general down regulation of energy metabolism in small adipocytes is further suggested by the finding that cytochrome C, citrate synthase, pyruvate dehydrogenase, xanthine dehydrogenase protein levels are all decreased at the protein level in small adipocytes. Clathrin protein expression was also decreased in small adipocytes from FIRKO and control mice. This is may also be important in the metabolic phenotype, since clathrin has been shown to be involved in intracellular vesicle transport including GLUT4 (19) and insulin receptor (20) endocytosis. Furthermore, subcellular distribution of clathrin has been shown to be regulated by insulin (21).

Besides these proteins that are involved in adipocyte metabolism, we found differential expression of several proteins not previously related specifically to adipocyte function. Annexin II and cyclophilin A modulate the transport of cholesterol ester from caveolae to internal membranes as part of a caveolin-annexin II lipid-protein complex (22). Both annexin II and cyclophilin A proteins levels were decreased in small adipocytes, and this could contribute to a decreased substrate availability in small adipocytes. Carbonic anhydrase 3 (CA 3), which is rich in skeletal muscle and adipocytes, was decreased at the protein level in small adipocytes. CA 3 has been shown to be the most abundant protein in rat adipocytes (23). The observation that the concentration of this enzyme decreases in the Zucker fatty rat with obesity and insulin resistance (23) is contrary to with our finding that large adipocytes have higher levels of CA 3 but are more insulin resistant than small adipocytes (3). Clearly insulin sensitivity is determined by many factors, and that the specific role of CA 3 needs to be determined.
Protein levels of glucose regulated protein (GRP) 58, which is involved in the STAT signal transduction in response to growth factors and cytokines (24); are also lower in small FIRKO adipocytes. It is possible that this produces alterations in growth factor and cytokine signaling which contribute to these differences heterogeneity in adipocyte size. Whether the lower levels of CA 3, annexin II and cyclophilin A protein expression represent intrinsic differences contributing to adipocyte heterogeneity or are secondary to adipocyte heterogeneity needs to be further investigated.

In addition to the differences in protein expression dependent on adipocyte size, our data also indicate differences in protein expression due to a loss of insulin signaling in adipocytes from FIRKO mice. The major changes detected in these cells which lack insulin signaling were decreases in levels of several proteins involved in lipid and energy metabolism, including the fatty acid translocase CD 36, succinyl CoA transferase, and methyl malonate semialdehyde dehydrogenase. This suggests that the expression of these proteins is directly regulated by insulin, and this is confirmed by the finding of parallel regulation at the mRNA level. The finding that elongation factor 2 protein is also decreased in both small and large FIRKO adipocytes is in accordance with the observation that insulin rapidly induces the biosynthesis of elongation factor 2 (25). EH domain containing protein 2 is also decreased in FIRKO fat cells. This protein is known to be involved in membrane dynamics and growth factor signaling and could therefore play a role in adipocyte differentiation processes (20-26).

An opposite pattern with increased protein expression in FIRKO adipocytes was observed for vimentin, GRP 78 (HSP 70), aldehyde dehydrogenase 2 and transketolase, suggesting that insulin negatively regulates the expression of these proteins. Vimentin has
been considered an early marker of adipogenesis, since vimentin regulates lipid droplet content during differentiation (26,27). Moreover, vimentin was identified by mass spectrometry in intracellular GLUT4-enriched membranes, suggesting a role of this protein in glucose transport (28). Immunoelectron microscopy of the GLUT4-containing membranes also revealed their association with these cytoskeletal proteins. Disruption of intermediate filaments and microtubules in 3T3-L1 adipocytes by microinjection of a vimentin-derived peptide causes dispersion of perinuclear GLUT4 to peripheral regions of the cells (28). Thus, the increased vimentin in FIRKO cells could contribute to altered trafficking of glucose transporters (3) leading to heterogeneity of adipocyte size in FIRKO mice. GRP 78 belongs to the heat shock protein HSP 70 family and is expressed during early organogenesis (29). Moreover, although no direct insulin effect could be detected, GRP 78 expression has been shown to be regulated similarly to GLUT1 in response to stress (30), and in our previous studies western blotting for candidate proteins, we showed that GLUT1 protein levels were markedly decreased in both large and small FIRKO adipocytes as compared to controls.

In conclusion, the knockout of the insulin receptor in fat appears to unmask an intrinsic heterogeneity in adipocytes accompanied by changes in cell size, and mRNA and protein expression patterns. Using a proteomics approach, we have identified multiple differentially expressed proteins that participate in these alterations, including proteins that involved in the regulation of adipocyte differentiation, triglyceride storage and adipocyte metabolism. Approximately half of the changes observed at the protein level would have been undetected by mRNA analysis, and others were detected only by fraction of cells into specific compartments. These protein alterations provide insight
into the nature of adipocyte heterogeneity and could represent potential novel therapeutic
targets for modulation of adipose mass and the treatment or prevention of obesity.
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Figure Legends

Figure 1. Experimental design. Heterogeneity of white adipose cell size in FIRKO mice is illustrated by hematoxylin and eosin staining of white adipose tissue sections from random-fed, 3 month-old male FIRKO and IR lox/lox mice (10X). Adipocytes from epididymal fat pads of pooled FIRKO and IR lox/lox mice were isolated by collagenase digestion and separated by filtration through a 75 µm pore size mesh nylon screen. Small (diameter < 75 µm) and large (diameter > 75 µm) adipocytes were then fractionated into cytosol and membranes, and the membrane further fractionated into loosely associated membrane proteins (KCl extracted fraction) and integral or tightly associated membrane proteins (CHAPS fraction) as described in Methods. 100 µg of total protein of each extract was loaded on to a 1.8 ml 10-30% sucrose gradient then centrifuged at 35,000 rpm for 20 hr at 4°C. The resultant gradient was divided into ten 200 µl fractions from the top to bottom of the tube and each of these was analyzed on 7.5-15% SDS-PAGE gels. Proteins resolved by SDS-PAGE were visualized by Sypro Ruby stain. For differentially expressed proteins, bands were excised from the polyacrylamide gel and tryptically digested in-gel. The samples were analyzed by either electrospray ion trap mass spectrometry (ESI) or matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF).

Figure 2. Differential protein expression in small and large adipocytes of FIRKO (FIRKO S and L) and IR lox/lox mice (IR lox S and L). Adipocytes were isolated and fractionated as described in the legend to Figure 1. The gradient fractions from
adipocytes of four IR lox/lox and three FIRKO were resolved side-by-side on a 7.5-15% polyacrylamide gel and stained with Sypro Ruby. Bands (marked by rectangles) that exhibited different protein expression between small and large adipocytes from FIRKO and IR lox/lox mice were excised from the gel, and after digestion analyzed by mass spectrometry as described in Methods. Non-muscle myosin, heavy chain, form A was only detected in large adipocytes from both FIRKO and IR lox/lox adipocytes. A representative Western blot and quantitation of the data (mean± SEM) from at least three independent experiments confirmed the proteomics result of exclusive expression of this non-muscle myosin, heavy chain in large adipocytes within the limit of detectability.

Figure 3. Heterogeneity of protein expression patterns depending on the adipocyte size. Differential protein expression in isolated adipocytes depending on their diameter from 3 month-old male IR (lox/lox) and FIRKO mice. Adipocytes from epididymal fat pads of four IR lox/lox and eight FIRKO mice were isolated by digestion with collagenase I, pooled, and separated into two different subsets of small (IR lox S, FIRKO S) and large (IR lox L, FIRKO L) adipocytes using a nylon mesh of 75 µm pore size. Three patterns of differential protein expression in small and large adipocytes from FIRKO and IR lox/lox mice were observed: A) Decreased levels in small adipocytes of both FIRKO and IR lox/lox mice (CPT 2, aP2, clathrin heavy chain, fatty acid synthase); B) Decreased levels only in small FIRKO adipocytes, with indistinguishable protein levels between small and large IR lox/lox adipocytes (annexin A6, HSP 47, HSP 60); C) Decreased protein levels only in small IR lox/lox adipocytes, with indistinguishable protein expression in small and large FIRKO adipocytes (annexin 2). For each of the
three patterns a representative Western blot and quantitative analysis (mean ± SEM) from at least three independent experiments are shown.

**Figure 4. Differential protein expression in isolated adipocytes in 3 month-old male FIRKO and IR lox/lox mice independently from the adipocyte diameter.** Adipocytes from epididymal fat pads of four IR lox/lox and eight FIRKO mice were isolated and fractionated as described in Figure 3. Two patterns of different protein expression in small and large FIRKO and small and large IR lox/lox mice were observed: 

**A)** Decreased protein expression in both small and large FIRKO adipocytes as compared to small and large IR lox/lox adipocytes (EH domain containing protein 2, CD36, elongation factor 2); 

**B)** Decreased protein expression in both small and large IR lox/lox adipocytes as compared to FIRKO adipocytes in both cell size groups (vimentin, GRP 78). For each of the three patterns a representative Western blot and quantitation of the data (mean ± SEM) from at least three independent experiments are shown.
Figure 1

FIRKO → Adipocyte isolation → Filtering of adipocyte suspension (75 μm nylon mesh)

- FIRKO large
- FIRKO small
- IR lox large
- IR lox small

Protein extraction and subfractionation

Membrane fraction
- KCl fraction
- Ionic bound membrane proteins

Cytosol fraction
- Chaps fraction
- Integrated membrane proteins

100 μg total protein to a 10-30% sucrose gradient, 20 h ultra centrifugation

100 μl sucrose gradient fractions

- Fixation
- Staining
- Cut bands of interest

7-15% SDS Gel

Mass Spectrometry
Figure 2

A. Myosin, Heavy chain, nonmuscle form A

B. Relative units

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Figure 3

A. CPT 2, eP2, Clathrin HC, FAS

Others:
Carbonic anhydrase 3
Cyclophilin A

Annexin A6, HSP 47, HSP 60

Others:
Acyl CoA DH
Citrate Synthase
Acetyl CoA DH
Xanthine DH
Cytochrome C
GRP 58

C. Annexin 2

Others:
Pyruvate DH
Figure 4

A. 

EH domain containing protein 2

FA translocase, CD 36

Elongation factor 2

Vimentin

GRP 78

Others:
Succinyl CoA transferase
Methylmalonate
Seetaldehyde DH

B. 

Others:
Aldehyde DH 2
Transketolase
Polymerase I and
transport release factor
Role of insulin action and cell size on protein expression patterns in adipocytes
Matthias Bluher, Leanne Wilson-Fritch, John Leszyk, Palle G. Laustsen, Siliva Corvera and C. Ronald Kahn

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