Terminal Galactosylation and Sialylation Switching on Membrane Glycoproteins upon TNF-Alpha-Induced Insulin Resistance in Adipocytes

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Insulin resistance (IR) is a complex pathophysiological state that arises from both environmental and genetic perturbations and leads to a variety of diseases, including type-2 diabetes (T2D). Obesity is associated with enhanced adipose tissue inflammation, which may play a role in disease progression. Inflammation modulates protein glycosylation in a variety of cell types, and this has been associated with biological dysregulation. Here, we have examined the effects of an inflammatory insult on protein glycosylation in adipocytes. We performed quantitative N-glycome profiling of membrane proteins derived from mouse 3T3-L1 adipocytes that had been incubated with or without the proinflammatory cytokine TNF-alpha to induce IR. We identified the regulation of specific terminal N-glycan epitopes, including an increase in terminal di-galactose- and a decrease in biantennary alpha-2,3-sialylglycans. The altered N-glycosylation of TNF-alpha-treated adipocytes correlated with the regulation of specific glycosyltransferases, including the up-regulation of B4GalT5 and Ggta1 galactosyltransferases and down-regulation of ST3GalI sialyltransferase. Knockdown of B4GalT5 down-regulated the terminal di-galactose regulation of ST3Gal6 sialyltransferase. Knockdown of B4GalT5 down-regulated the terminal di-galactose N-glycans, confirming the involvement of this enzyme in the TNF-alpha-regulated N-glycome. SILAC-based quantitative glycoproteomics of enriched N-glycopeptides with and without deglycosylation were used to identify the protein and glycosylation sites modified with these regulated N-glycans. The combined proteome and glycoproteome workflow provided a relative quantification of changes in protein abundance versus N-glycosylation occupancy versus site-specific N-glycans on a proteome-wide level. This revealed the modulation of N-glycosylation on specific proteins in IR, including those previously associated with insulin-stimulated GLUT4 trafficking to the plasma membrane. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.054221, 141–153, 2016.

The pathogenesis of type-2 diabetes (T2D) is a complex process driven by the failure of tissues to respond to insulin (insulin resistance; IR) and the subsequent loss of insulin secreting pancreatic beta-cells. IR has become a significant health problem, and the etiology of IR is multifaceted and dynamic. IR manifests in multiple tissues, including muscle, liver, pancreas, and brain but there is increasing evidence to support a central role of adipose tissue as a major contributor to whole body IR (1). Adipose tissue is the central site of lipid storage and important for the regulation of circulating fatty acids but is also a key regulator of whole body carbohydrate metabolism through its function as an endocrine organ. It is therefore not surprising that disruptions to normal adipose biology can have serious consequences on whole body energy metabolism. For example, artificial induction of IR in adipose tissue using adipose-specific GLUT4 glucose transporter knockout mice also resulted in liver and muscle IR and whole body glucose intolerance and hyperinsulinemia (2). Adipose tissue has a remarkable ability to rapidly remodel through both hypertrophy and hyperplasia, whereas excessive expansion, as occurs in obesity, has been associated with hypoxia, inflammation, and cell death. Obesity is often associated with elevated levels of TNF-alpha and other pro-inflammatory cytokines, and incubation of adipocytes with TNF-alpha has been shown to cause IR (3).

Proinflammatory cytokines such as TNF-alpha are primarily secreted from adipose tissue by resident and infiltrating macrophages, and the number of macrophages, along with other types of immune cells, increases in obese adipose tissue (4–6). Upon binding to cell surface receptors, TNF-alpha stimulates signaling pathways, which activate several transcriptional regulators, including nuclear factor-kappa B and nuclear factor of activated T-cells (7). The signaling and transcriptional effects of TNF-alpha modulate several biolog-
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...ical processes in adipocytes, including lipid and glucose metabolism, adipokine production, cellular stress, and calcium transport. Sustained modulation of these processes induce IR through several mechanisms, including reduced peroxisome proliferator-activator receptor (PPAR)–γ activity, reduced expression of GLUT4 (8), reduced expression and activity of proteins regulating insulin-signaling (9–11), production of deleterious ceramide species (12), and the production of reactive oxygen species, all of which contribute to endoplasmic reticulum (ER) and mitochondrial stress (13, 14). TNF-alpha has been reported to modulate the expression of hundreds of genes (15), and it is highly likely the above processes jointly induce insulin sensitivity in a combinatorial fashion.

Modulation of enzymatic N- and O-linked protein glycosylation has been documented under a variety of proinflammatory conditions (16) and has been correlated with altered expression of several glycosyltransferases involved in forming terminal glycopeptides, including sialylation, galactosylation, and fucosylation. For example, TNF-alpha modulates the expression of alpha2,3-sialyltransferases in a range of cells and tissues (17–20) resulting in an increase in the sialyl-LewisX epitope displayed on glycoproteins. There is growing evidence for a direct link between protein glycosylation and the pathogenesis of T2D. For example, islets from humans with T2D show reduced expression of MgaT4a mRNA in pancreatic beta cells (21). In mice, MgaT4a codes for an enzyme responsible for the beta1,4-GlcNAc branching on the GLUT2 glucose transporter, which has been associated with its cell-surface expression and glucose-stimulated insulin secretion (22).

Given the documented modulation of multiple glycosyltransferases under proinflammatory conditions and evidence supporting a role for glycosylation in insulin secretion, we hypothesized that prolonged exposure to inflammatory adipokines would alter the adipocyte N-glycosylation. To investigate this hypothesis, we performed a systems-wide analysis of N-linked glycosylation in adipocytes treated with TNF-alpha to induce IR. We integrated N-glycome profiling with quantitative N-glycoproteomic workflows to site specifically quantify the regulated glycosylation on a proteome-wide level.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—3T3-L1 mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 2 mM l-alanyl-l-glutamine in 10% CO_2 at 37 °C. Confluent cells were differentiated into adipocytes as previously described (23) and used 10–12 days after differentiation. For quantitative proteomic experiments, cells were 2-plex SILAC labeled with ^13C_6-^15N_2-lysine and ^13C_6-^15N_4-arginine as previously described (24). Chronic low-dose inflammation-induced insulin resistance was mimicked by incubation with TNF-alpha (2 ng/ml) for 4 days and media changed every 24 h. For knockdown experiments, 3T3-L1 adipocytes at day 7 postdifferentiation were trypsinized and resuspended in Cell Line Nucleofector Buffer L (Lonza, Switzerland) containing 200 nM of scramble siRNA (5'-uucuggaaggauuacgutt) or pooled anti-B4GalT5 siRNA (5'-gggccgaugauuacacatt, 5'-ggac- uggauugggaagutt, 5'-gaccuucuccuggaagatt) (GenePharma, China). Cells were electroporated using the A-033 program with the Amaxa Nucleofector (Lonza) and plated onto matrigel-covered plates. Cells were assayed 72 h following electroporation.

3H-2-deoxyglucose Uptake—Differentiated 3T3-L1 adipocytes were serum starved in DMEM for 2 h, washed briefly with PBS, and incubated in Krebs ringer buffer (0.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 120 mM NaCl, 6 mM KC1, 1 mM CaCl_2, 1.2 mM MgSO_4, and 12.5 mM HEPES (pH 7.4)) containing 0.2% BSA at 37 °C for 15 min. Cells were stimulated with 100 nM insulin for 20 min. To determine nonspecific glucose uptake, 25 μM cytochalasin B was added to a control wells before addition of 3H-2-deoxyglucose. During the final 5 min of insulin stimulation, 3H-2-deoxyglucose transport was initiated by addition of 3H-2-deoxyglucose (PerkinElmer). Following three rapid washes in ice-cold PBS, cells were solubilized in 1% (w/v) Triton X-100 in PBS on a shaker for 1 h and assessed for radioactivity by scintillation counting using a β-scintillation counter. All experiments and subsequent data handling were normalized to protein amount as determined using BCA assay.

(Glyco)peptide Sample Preparation—3T3-L1 adipocytes were washed briefly in ice-cold PBS and lysed in 100 mM sodium carbonate containing a protease inhibitor mixture (Roche) by tip-probe sonication. The lysate was rotated at 4 °C for 30 min and centrifuged at 100,000 × g for 90 min at 4 °C. The microsomal pellet was resuspended in 6 mM urea, 2 mM thiourea, 25 mM trichloroacetic acid, 1% SDS (pH 7.5), and membrane-associated proteins precipitated using chloroform/methanol/water (1:4:3). Proteins were resuspended in 6 mM urea, 2 mM thiourea, and 25 mM trichloroacetic acid (pH 7.5). Quantification was performed with Qubit fluorosence and 2.2 mg light- and heavy-labeled protein mixed followed by reduction with 10 mM DTT at room temperature for 60 min and alkylation with 25 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. The reaction was quenched with 10 mM DTT and digested with LysC (1:50 enzyme:substrate; Wako, Japan) for 2 h at room temperature. The mixture was diluted 1:5 with 25 mM triethylammonium bicarbonate (pH 7.5) and further digested with trypsin (1:50 enzyme:substrate; Promega) for 12 h at 32 °C. The peptide mixture was acidified to a final concentration of 2% formic acid and centrifuged at 16,000 × g for 10 min at room temperature. Peptides were desalted using hydrophilic-lipophilic balance solid-phase extraction cartridges washed with 5% acetoinure (MeCN), 0.1% trifluoroacetic acid (TFA) and eluted with 80% MeCN, 1% TFA. Approximately 20 μg out of the 4.4 mg of desalted peptide was removed and dried by vacuum centrifugation for total proteome analysis. The remainder of the –4.4 mg of desalted peptide was loaded directly onto an in-house made zic-hydrophilic interaction liquid chromatography solid phase extraction (HILIC SPE) column to enrich N-glycopeptides as previously described (25). The column was washed with 80% MeCN, 1% TFA, and enriched glycopeptides eluted with 0.1% TFA followed by 200 mM ammonium bicarbonate and concentrated with vacuum centrifugation. The 20 μl aliquot of peptide for the total proteome analysis was fractionated as previously described (26) using an in-house packed 320 μm × 20 cm column (3 μm particle size, amide-80 HILIC; Tosoh, Japan) into 12 fractions using an Agilent 1260 with a gradient of 90–60% MeCN containing 0.1% TFA over 40 min at 6 μl/min. One-third of each fraction was treated with 100 U of glycerol-free N-glycosidase F (PNGase F) for 16 h at 37 °C (New England Biolabs).

(Glyco)peptide Mass Spectrometry—The analysis of peptides for total proteome analysis and deglycosylated peptides was performed...
on a Dionex 3500RS coupled to a Q-Exactive Plus with Tune v2.4.1824 in positive polarity mode. Peptides were separated using an in-house packed 75 μm × 50 cm packed column (1.9 μm particle size, C18AQ; Dr Maisch, Germany) with a gradient of 2–30% MeCN containing 0.1% formic acid over 120 min at 250 nl/min at 55 °C. An MS1 scan was acquired from 350–1550 m/z (70,000 resolution, 3e6 AGC, 100 ms injection time) followed by MS/MS data-dependent acquisition of the 20 most intense ions with higher collision dissociation (HCD) (17,500 resolution, 1e6 AGC, 60 ms injection time, 27 normalized collision energy (NCE), 1.2 m/z isolation width).

The analysis of glycopeptides was performed on a Dionex 3500RS coupled to an Orbitrap Fusion with Tune v1.2.1149 in positive mode. Glycopeptides were separated using an in-house packed 75 μm × 50 cm packed column (1.9 μm particle size, C18AQ; Dr Maisch, Germany) with a gradient of 2–30% MeCN containing 0.1% formic acid over 120 min at 250 nl/min at 55 °C. An MS1 scan was acquired from 550–1750 (120,000 resolution, 5e5 AGC, 100 ms injection time) followed by MS/MS spectrum. The reisolated precursor ion was subjected to both electron transfer higher collision dissociation (ETdCD) and collision induced dissociation (CID) MS/MS analysis (28–30). ETdCD MS/MS analysis was detected in the Orbitrap (30,000 resolution, 2e5 AGC, 200 ms injection time, 40 NCE, 2.0 m/z quadrupole isolation width). The acquisition strategy included a product ion triggered re-isolation of the precursor ion if HexNAc oxonium ions (138.0545 and 204.0867 m/z) were detected among the top 20 fragment ions of the HCD and detection in the Orbitrap (30,000 resolution, 2e5 AGC, 200 ms injection time, 40 NCE, 2.0 m/z quadrupole isolation width). The precursor tolerance was set to 20 ppm with a maximum of two full trypsin miss cleavages. The peptides were searched with 2-plex Arg0/Lys0 and Arg10/Lys8, oxidation of methionine, and deamination of asparagine and glutamine set as variable modifications while carbamidomethylation of cysteine was set as a fixed modification. A precursor isotope off set was enabled (narrow) to account for incorrect precursor monoisotopic reporting (< 1.0 Da). All data were searched as a single batch with peptide spectral match FDR set to 1% using the peptide spectral match Validator node and a minimum Byonic score of 100 was applied. Only HCD MS/MS spectra containing HexNAc oxonium ions within the range of 138.05–138.06 and 204.08–204.09 m/z were annotated in the final list of glycopeptides as previously described (27). Localization of glycosylation sites in the ETdCD data was performed manually. Identified glycopeptides were quantified using the Precursor Ions Quantifier node. Event detection was set to 2 ppm to generate extracted ion chromatograms with a retention time tolerance of isotope pattern multiplets set to 0.2 min.

**Released N-glycan Sample Preparation—**N-glycome profiling was performed essentially as described previously (34). Briefly, 10 μg of membrane-associated proteins were dot-blotted onto PVDF membranes and allowed to dry overnight. The membranes were stained briefly with direct blue 71 in 40% ethanol containing 10% acetic acid and washed with water. Immobilized proteins were excised and the membrane blocked with 1% polyvinyl pyrrolidone 4000 for 5 min followed by washing with water. For alpha- and beta-exogalactosidase treatments, immobilized proteins were treated with 4 U of alpha-exogalactosidase (alpha1–3,6), 4 U of beta-exogalactosidase (beta1–3), 8 U of beta-exogalactosidase (beta1–4) or a mixture of 4 U, and 8 U of beta1–3 and beta1–4, respectively. N-glycans were released with 2.5 U of PNGase F for 16 h at 37 °C. Released glycans were collected, incubated with 100 mM ammonium acetate (pH 5.0) for 1 h at 23 °C and dried by vacuum centrifugation. N-glycans were reduced with 1 μl NaBH4 in 50 mM KOH for 3 h at 50 °C and desalted using in-house packed cation-exchange microcolumns (Dowex, Midland, MI) according to the manufacturer’s instructions and dried by vacuum centrifugation. Residual borate was removed with 1% acetic acid in methanol and dried by vacuum centrifugation.

**Released N-glycan Mass Spectrometry and Data Analysis—**The N-glycans were profiled on an Agilent 1100 coupled directly to an Agilent MSD ion trap XCT Plus instrument in negative polarity mode. Glycans were separated on a 200 μm × 10 cm porous graphitized carbon (PGC) column (5 μm particle size, hypercarb; Thermo Scientific) with a gradient of 2–16% MeCN containing 10 mM NH4HCO3 over 20 min at 2 μl/min. MS1 scans were acquired from 250–2200 m/z (7e4 AGC, 100 ms injection time) followed by MS/MS data-dependent acquisition of the three most intense ions with CID (MS/MS fragment ion mass range ± 1 V, small fragment matching = enabled, and ramp amplitude = 30–200%). Monoasaccharide compositions of the N-glycans were determined from the precursor masses (200 ppm mass tolerance) using the GlycoMod tool (http://web.expasy.org/glycomod/), CID MS/MS and PGC LG retention time by comparing to in-house datasets and a public glycan database (UnicarbKB). Alpha-2,3-linked and alpha-2,6-linked sialylated N-glycans were differenti-
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ated by PGC-LC retention time (alpha-2,6 < alpha-2,3). The CID MS/MS spectra were manually annotated, which yielded structural information such as glycan topology, sialic acid and fucose linkage type, and isobaric isomer formation (34). MS/MS fragment ion masses and general knowledge of the mammalian N-glycosylation were used to assign some of structural aspects, yet some structural ambiguity remained in a subset of the reported N-glycans as indicated with brackets. The extracted ion chromatograms (XIC) (m/z ± 0.5) were derived for all observed charge states of each identified N-glycan containing MS/MS and used to determine the relative N-glycome distribution.

Quantitative-PCR—RNA was extracted using Trizol/chloroform (5:1) and precipitated with isopropanol followed by 70% ethanol. The RNA was quantified with NanoDrop (Thermo Scientific) and 10 μg reverse transcribed into cDNA with the Omniscript RT kit (Qiagen, Netherlands) according to the manufacturer’s instructions for 60 min at 37 °C. Quantitative-PCR was performed using the LightCycler 480 Real-Time PCR 480 system (Roche Applied Science, Germany) using the Universal Probe Master system. The following primer sequences and probes were used: B4GalT5 (NM_019835.2) forward: 5′-aatcactagccttcataagg and reverse: 5′-gcctgcaattcttcctgt with probe number 45; ST3GaL6 (NM_018784.2) forward: 5′-gaggccctgcatccctcctc and reverse: 5′-aggtctccacagttgga with probe number 91; Ggt1 (NM_001145821.1) forward: 5′-gaagctcggctcctcag and reverse: 5′-ctctggcctgcctaagga; and Man1a (NM_0088548.4) forward: 5′-aagacctcgcgctctcag and reverse: 5′-gattcttgtaggcgctcgc and reverse: 5′-ccacagctggtgcctc with probe number 4. All data were normalized to cyclophilin B mRNA, and the relative mRNA levels were determined using the comparative threshold cycle (delta-Ct) method (35).

Experimental Design and Statistical Rationale—All presented data show a minimum of three independent biological replicates. For 3H2-deoxyglucose uptake assays and Western blotting, four biological replicates were performed. For N-glycomic analysis, three biological replicates were performed with two LC-MS/MS technical replicates. For SILAC analysis, two biological replicates were performed with “forward” labeling i.e. Arg0/Lys0 = control; Arg10/Lys8 = TNF treatment and a third replicate was performed with “reverse” labeling i.e. Arg0/Lys0 = TNF treatment; Arg10/Lys8 = control. For O-QPCR analysis, three biological and three technical replicates were performed. Moderated t-tests adjusted for multiple testing with Benjamini Hochberg FDR analysis were used to determine significantly regulated N-glycans (minimum of three biological replicates), proteins (minimum of three biological replicates), deglycosylated peptides (minimum of two biological replicates), and glycopeptides (minimum of two biological replicates). Significant regulation was determined using an adjusted p value <0.05 unless otherwise stated.

RESULTS

TNF-alpha Induced IR Modulates the N-glycome of Adipocytes—Treatment of differentiated 3T3-L1 mouse adipocytes with TNF-alpha caused a marked reduction in insulin-stimulated 2-deoxyglucose uptake (p < 0.005; t test), a functional consequence of IR, as previously described (Fig. 1A)(13). This was associated with a decrease in Akt expression and insulin-signaling responses at the level of Akt active site phosphorylation at Thr-308 (p < 0.05; t test)(Fig. 1B). Released N-glycans from membrane-associated proteins from adipocytes treated with or without TNF-alpha were profiled by PGC-LC-MS/MS. In total, 87 unique N-glycans were identified at the monosaccharide compositional level based on precursor mass (Supplemental Table S1). Using PGC-LC retention time and MS/MS, a total of 43 glycan structures were assigned. Six N-glycans could arise from different isobaric compositions (i.e. the combination of NeuAc and hexose has the same mass as NeuGc and fucose). However, none of these N-glycans were confirmed by MS/MS. The 43 N-glycan structures confirmed by retention time and MS/MS were quantified by XIC-LFQ. Two of the seven high-mannose-containing N-glycans were significantly altered in abundance including an increase in HexNAc2HexS and a decrease in HexNAc2Hex4 (adjusted p < 0.05; δ t test)(Fig. 1C). Thirty-six hybrid/complex type N-glycans were quantified, which revealed the regulation of six N-glycans, including significant increases in the truncated complex N-glycan, HexNAc2HexFuc, (adjusted p < 0.05; t test) and an increase in three complex N-glycans containing an unspecified terminal hexose residue (galactose, mannose or glucose, all 162 Da) linked to the penultimate beta-galactose at the nonreducing end of the glycan (adjusted p < 0.05, t test)(Fig. 1D). Treating adipocyte enriched membrane proteins with an alpha-exogalactosidase (alpha1-3,6) followed by analysis of released N-glycans by PGC-LC-MS/MS resulted in a significant reduction in the core-fucosylated non-sialylated di-hexose containing N-glycans, HexNAc2Hex6Fuc1 and HexNAc2Hex4Fuc1 (adjusted p < 0.05, t test)(Supplemental Fig. S1). Similarly, treatment of adipocyte enriched membrane proteins with beta-exogalactosidases (beta1-3, beta1-4 or a mixture of beta1-3,4) reduced the core-fucosylated non-sialylated di-hexose containing N-glycan HexNAc4Hex2Fuc1 (Supplemental Fig. S1). These results indicate the presence of a mixture of alpha- and beta-terminating galactose residues on N-glycans that were increased with TNF-alpha treatment. In contrast, the N-glycan, HexNAc2Hex6Fuc1NeuAc2, containing two terminal alpha-2,3-linked sialic acid residues, was significantly decreased in relative abundance upon TNF-alpha treatment (adjusted p < 0.05, t test) (Fig. 1D). The summed XIC areas of the N-glycans containing the terminal di-galactose moiety appeared to be increased while the summed XIC areas of all the sialylated N-glycans appeared to be decreased with TNF-alpha treatment, although neither of these changes reached significance (p = 0.06 and p = 0.08, t test)(Figs. 1E and 1F).

PGC-LC is able to separate alpha-2,3-linked and alpha-2,6-linked sialylated N-glycans, which revealed the summed XIC areas of allalpha-2,3-terminating sialylated N-glycans, including significant increases (p < 0.08, t test)(Supplemental Fig. S1). These results suggest there is a reciprocal regulation between terminal galactosylation and alpha-2,3-sialylation in mouse 3T3-L1 adipocytes upon TNF-alpha-induced IR.

A Combined Strategy for the Quantification of Glycosylation Enzymes and Site-Specific N-glycosylation—Analysis of the membrane N-glycome of adipocytes suggested that TNF-alpha modulates multiple glycosylation enzymes (glycosi-
dase/glycosyltransferases) to result in changes to $N\text{-linked}$ glycosylation. System-wide (glyco)proteomics workflows were established to identify and quantify glycosylation enzymes and to pinpoint specific glycosylation sites on membrane proteins containing these regulated $N\text{-glycans}$ in normal and IR adipocytes (Fig. 2). Differentiated 3T3-L1 adipocytes were 2-plex SILAC labeled with Arg0/Lys0 or Arg10/Lys8 and treated with either control or TNF-alpha in biological triplicate, including a label-switching replicate. Membrane-associated proteins were enriched and normalized prior to trypsin digestion. An aliquot of the digests were fractionated by amide-HILIC/HPLC and analyzed by nanoUHPLC-MS/MS on a Q-Exactive Plus mass spectrometer. Membrane-associated proteins, including glycosidases and glycosyltransferases, were identified and quantified with MaxQuant (31), followed by statistical analysis in Perseus. $N\text{-glycopeptides}$ were enriched from the remainder of the tryptic digests using zic-HILIC SPE and fractionated by neutral pH C18/HPLC. An aliquot of each enriched glycopeptide fraction was treated with PNGase F to release $N\text{-glycans}$ and the deglycosylated peptides analyzed by nanoUHPLC-MS/MS on a Q-Exactive Plus mass spectrometer. Formerly glycosylated peptides were identified by the presence of a deamidated Asn within the $N\text{-linked}$ glycosylation sequon (NxS/T/C, x ≠ P) and quantified with MaxQuant. This was used to build a database of peptide sequences known to be glycosylated to facilitate the subsequent analysis of intact glycopeptides. The remaining aliquot of the intact glycopeptide fraction was analyzed directly by nanoUHPLC-MS/MS on an Orbitrap Fusion mass spectrometer employing HCD and product-ion triggered EThcD/CID MS/MS (29, 30). In this approach, diagnostic glycan oxonium-ions present in the HCD MS/MS spectrum trigger the reisolation of the same precursor ion for a subsequent EThcD and CID MS/MS fragmentation. Peptides and their attached glycan monosaccharide compositions were quantified with Byonic in Proteome Discoverer, followed by statistical analysis in Perseus.

Quantification of the TNF-alpha Regulated Membrane-Associated Proteome Including Glycosyltransferases and Glyco-
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Fig. 2. Novel strategy for the quantification of glycosylation enzymes and site-specific N-glycosylation. Differentiated 3T3-L1 adipocytes were 2-plex SILAC labeled with Arg⁰/Lys⁰ or Arg¹⁰/Lys⁰ and treated with or without TNF-alpha in biological triplicate including a label-switching replicate. Membrane-associated proteins were enriched and digested with trypsin. An aliquot of the membrane enriched digests were fractionated by amide-HILIC μHPLC and analyzed by nanoUHPLC-MS/MS on a Q-Exactive Plus mass spectrometer and quantified by MaxQuant followed by statistical analysis in Perseus. N-glycopeptides were enriched from a second aliquot of the digest using zic-HILIC SPE and fractionated by neutral pH C18 μHPLC. An aliquot of each fraction was treated with PNGase F to release N-glycans and the former glycopeptides analyzed by nanoUHPLC-MS/MS on a Q-Exactive Plus mass spectrometer. Former glycopeptides were quantified with MaxQuant and SequestHT in Proteome Discoverer by the presence of a deamidated Asn, and a database of former glycopeptide sequences was created. The intact N-glycopeptides were analyzed directly by nanoUHPLC-MS/MS on an Orbitrap Fusion mass spectrometer employing HCD and product-ion-dependent ETHcD/CID fragmentation events. Peptides and their attached glycan compositions were quantified by Byonic in Proteome Discoverer followed by statistical analysis in Perseus.

sidases—Analysis of the membrane-associated proteome of differentiated 3T3-L1 adipocytes treated with or without TNF-alpha identified a total of 7,258 proteins with 6,259 quantified in all three biological replicates (Supplemental Table S2). In total, 213 proteins were identified as significantly regulated (adjusted \( p < .05 \) and ±1.5 fold-change) (Fig. 3A). A total of 4,642 proteins contained a membrane gene ontology cellular compartmentalization annotation, and of these, 686 and 570 proteins also contained Golgi or ER annotation, respectively (Fig. 3B; Supplemental Table S2). To gain an overview of the signaling pathways regulated with TNF-alpha treatment, pathway analysis was performed with Ingenuity Pathway analysis. This highlighted several immunology-associated pathways, including acute-phase signaling among others and, confirmed the up-regulation of previously characterized TNF-alpha regulated transcription factors RelB, nuclear factor-kappa B, and STAT2 (Fig. 3C; Supplemental Tables S3). Interestingly, several growth factor receptors were also regulated, including epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), and platelet-derived growth factor receptor (PDGFR). Furthermore, pathway analysis revealed an increase in several receptors involved in interleukin/toll-like receptor signaling. We next compared the regulated proteins to a collection of curated transcriptomic datasets containing TNF-alpha regulated genes (15, 36, 37). A total of 68 transcripts and proteins showed the same direction of regulation, providing evidence of altered expression of these previously described genes at the protein level (Fig. 3D). Given the comprehensive coverage and quantification of Golgi/ER annotated proteins, we next compared these proteins against a curated list of glycosyltransferases/glycosidases (http://glycoenzymes.ccr.cancer.gov). In total, 94 mouse glycosyltransferases/glycosidases were quantified in all three biological replicates with 5 proteins significantly up-regulated (adjusted \( p < .05 \) and ±1.5 fold-change): ST6GalNAc4, Ggta1, Man1a, Man2b, and B4GalT5 (Fig. 3E; Supplemental Table S4). One protein, ST3Gal6, was down-regulated by more than 1.5-fold, although this was not significant at a cut-off of adjusted \( p \) value \( .05 \) (adjusted \( p = .06 \)). However, due to the observed down-regulation of alpha-2,3-linked sialoglycans in the N-glycome profiling, ST3Gal6 responsible for catalyzing the formation of alpha-2,3-sialylation, was included in the further analysis. The regulation of key glycosyltransferases (Ggta1, B4GalT5, and ST3Gal6) and glycosidases (Man1a and Man2b), which correlated with the observed N-glycome changes, was next investigated by q-PCR. This validated the down- and up-regulation of ST3Gal6 and B4GalT5 mRNA, respectively, upon TNF-alpha treatment (\( p < .05 \), t test) (Fig. 3F). Man1a mRNA levels were not significantly regulated, and Ggta1 and Man2b produced very low signal-to-noise traces and were excluded from further analysis. We also interrogated the regulation of B4GalT4, Ggta1, and ST3Gal6 in an in vivo IR model. Montminy and colleagues subjected mice to 12 weeks chow or high-fat diet and performed a comparative transcriptomic analysis of white adipose tissue (GSE14363) (38). Consistent with our model of TNF-alpha-induced IR in cultured 3T3-L1 adipocytes, Montminy and colleagues found that ST3Gal6 and B4GalT5 were down- and
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up-regulated by 3.1- and 1.9-fold, respectively. Two independent probes for GtA1 gave low signal intensity with poor correlation. We were therefore not able to assess the regulation of this alpha-galactosyltransferase under insulin resistance conditions at the mRNA level.

We have shown that analysis of the membrane-associated proteome of 3T3-L1 adipocytes treated with TNF-alpha identified regulated glycosyltransferases that correlated with increased expression of the galactosyltransferases B4GalT5 and GtA1, and the down-regulation of alpha-2,3-linked sialic acid containing N-glycans. This inverse correlation. We were therefore not able to assess the regulation of this alpha-galactosyltransferase under insulin resistance conditions at the mRNA level.

We have shown that analysis of the membrane-associated proteome of 3T3-L1 adipocytes treated with TNF-alpha identified regulated glycosyltransferases that correlated with the altered N-glycan structures. Specifically, the up-regulation of alpha- and beta-linked di-galactose terminating N-glycans correlated with increased expression of the galactosyltransferases B4GalT5 and GtA1, and the down-regulation of alpha-2,3-linked sialic acid containing N-glycans correlated with decreased ST3Gal6 expression.

B4GalT5 Regulates Di-Galactose Containing N-glycans—The observed correlations between the regulated glycosylation enzymes and N-glycan structures in TNF-alpha treated adipocytes were further investigated by modulating the expression of the glycosyltransferases, followed by N-glycome profiling. 3T3-L1 adipocytes were electroporated with siRNAs targeting mouse B4GalT5, ST3Gal6, or a scramble sequence to act as a control. Significant knockdown of B4GalT5 mRNA was obtained (p < .01, t test)(Fig. 4A). However, we were unable to obtain significant knockdown of ST3Gal6 despite investigating three independent siRNA sequences (<20% knockdown). Therefore, an N-glycomic analysis of ST3Gal6 knockdown cells was not performed. As expected, knockdown of B4GalT5 did not significantly alter high-mannose structures (Fig. 4B). However, a significant reduction in a nonfucosylated N-glycan containing two terminal di-galactose residues (HexNAc$_2$Hex$_7$) was observed with B4GalT5 knockdown (p < .05, t test)(Fig. 4C). Three other N-glycans containing terminal di-galactose epitopes were similarly reduced upon B4GalT5 knockdown, although not significant (p = .06-.2)(Figs. 4D-4F). These data further suggest the increase in di-galactose terminating N-glycans observed during TNF-alpha-induced IR are mediated at least in part by B4GalT5.

Site-Specific N-glycopeptide Quantification of Membrane-Associated Proteins TNF-Alpha Induced IR—We next investigated the specific glycoproteins and glycosylation sites targeted for modification of these regulated N-glycans. This would potentially allow functional insights into the contribution of these N-glycans to IR. Analysis of zic-HILIC enriched and PNGase F deglycosylated peptides identified 2,187 unique peptides containing a deamidated Asn (Supplemental Table S5). This resulted in the quantification of 951 unique deamidation sites in the motif for N-linked glycosylation (NxS/T/C) in two out of three biological replicates (Supplemental Table S6). In total, 79 unique deamidated peptides on 50 proteins displayed significantly altered abundance with TNF-alpha treatment (adjusted p < .05 and 1.5-fold change). Measured changes in the abundance of these deamidated peptides may result from altered abundance of the protein itself, changes in N-glycan occupancy, or changes in in vivo deamidation levels. To investigate this, we correlated the fold-change of the total protein abundance to the deamidated peptide fold-change (Fig. 5A). Of the 50 proteins quantified with regulated deamidated peptides, total protein quantification was obtained for 48 proteins. Normalizing the fold-

Fig. 3. Quantification of the TNF-alpha regulated membrane-associated proteome in 3T3-L1 adipocytes. (A) Distribution of quantified proteins upon TNF-alpha treatment. (B) Selected gene ontology cellular compartmentalization of the quantified proteins. (C) TNF-alpha regulated receptors and transcription factors. (D) TNF-alpha regulated proteins correlating with curated transcriptomic datasets. (E) Quantification of glycosylation-associated proteins (glycosidases and glycosyltransferases) (yellow = adjusted p > .06; red = adjusted p < .06 and 1.5-fold) (F) Quantification of glycosidase/glycosyltransferase mRNA using Q-PCR in 3T3-L1 adipocytes treated with either control or TNF-alpha (2 ng/ul; 4 days). Results are normalized to cyclophilin B (n = 4 biological replicates, *p < .05, **p < .01, t test).
change of the deamidated peptide to the total protein levels resulted in the identification of only three deamidation sites that were >50% regulated compared with total protein levels: Asn-51 on SPPL2A, Asn-947 on COL18A1, and Asn-280 on H2-D1. The deamidation site on COL18A1 was N-terminal to a Gly residue that has previously been shown to increase the kinetics of deamidation and may reflect changes in deamidation rather than N-glycan occupancy (39). Taken together, these data suggest the relative N-glycan occupancy of adipocyte membrane proteins subjected to TNF-alpha-induced IR remains largely unchanged, and the majority of regulated deamidated peptides result from changes in total protein levels.

Next, a concatenated peptide database containing the 2,187 unique deamidated Asn-containing peptide sequences from 1,041 proteins was created and used to search the intact glycopeptides analyzed by HCD and EThcD MS/MS spectra using Byonic. This search was performed with an N-glycan database containing the masses of 309 different mammalian N-glycan compositions. The analysis of the HCD and EThcD MS/MS data resulted in the identification of 1,563 and 291 unique N-glycopeptides, respectively (Supplemental Table S7). A combined total of 1,580 unique N-glycopeptides were identified that covered 332 unique peptide sequences on 154 proteins and carrying a total of 121 unique glycan monosaccharide compositions. At least one of the 87 glycan compositions initially identified by PGC-LC-MS/MS N-glycan profiling was identified on one or more peptide sequence. The 32 additional N-glycan compositions found on peptides that had not been identified by released N-glycome profiling with PGC-LC-MS/MS were identified on peptides with low abundance and included very small glycans (<3 monosaccharide residues) that have poor PGC retention or were large glycans (>11 monosaccharide residues) that may be difficult to profile due to high PGC retention and reduced ionization efficiency (40). Of the 1,580 nonredundant N-glycopeptides identified, 197 contained one of the six potential isobaric glycan compositions identified in the N-glycome analysis consisting of either a NeuAc and hexose or a NeuGc and fucose. We next interrogated the HCD MS/MS spectra for the presence of
Fig. 5. N-glycopeptide quantification of TNF-alpha induced IR in 3T3-L1 adipocytes. (A) Correlation of TNF-alpha regulated peptides containing a deamidated Asn within the N-linked glycosylation sequon (Nxs/T/C) compared with total protein levels ($n =$ at least 2/3 biological replicates; yellow $= $ adjusted $p < .05$ and ±1.5-fold but <50% regulated compared with total protein levels; red $= $ adjusted $p < .05$ and ±1.5-fold but >50% regulated compared with total protein levels). (B) Correlation of TNF-alpha regulated glycopeptides compared with total protein levels ($n =$ at least 2/3 biological replicates; yellow $= $ adjusted $p < .05$ and ±1.5-fold but <50% regulated compared with total protein levels; red $= $ adjusted $p < .05$ and ±1.5-fold but >50% regulated compared with total protein levels). (C) Quantification of glycopeptides spanning 12 glycosites from prolowl-density lipoprotein receptor-related protein 1 (LRP1) ($n =$ at least 2/3 biological replicates). (D) Annotation of two N-linked glycopeptides from LRP1 showing the HCD MS/MS and subsequent EThcD and CID MS/MS. All annotated ions are within 20 ppm tolerance for HCD and EThcD MS/MS and, 0.6 Da for CID MS/MS.
In a supplemental activation of NCE is dominated by c-

had been characterized in the localization and CID for confirmation of the glycan structure that EThcD for sequence identification and glycosylation site localization of two glycopeptides from LRP1 using HCD and terminal di-galactose epitopes. Figure 5 monosaccharide compositions were identified in the Thy1 and HexNAcHex5FucNeuGc at Asn-312 on Aco3. Five additional glycopeptides containing nonisobaric NeuGc were identified and interestingly, Asn-312 on Aco3 carried three additional glycans bearing NeuGc. These data combined with the N-glycome data suggest that NeuGc is extremely low in abundance in 3T3-L1 adipocytes.

Of the 1,580 N-glycopeptides identified, 883 were quantified in two out of three biological replicates. In total, 56 intact N-glycopeptides on 21 proteins were significantly regulated by TNF-alpha treatment (adjusted p < .05 and ±1.5-fold) (Supplemental Table S8). The regulation of these N-glycopeptides may arise from either changes in the abundance of the protein itself or the regulation of specific glycan compositions. To investigate this, we correlated the N-glycopeptide fold-changes to the relative total protein levels upon TNF-alpha treatment (Fig. 5B). Of the 21 proteins containing altered N-glycopeptides, total protein quantification was obtained for 20 proteins. Normalizing the N-glycopeptide fold-change to the total protein fold-change highlighted 16 that were more than >50% regulated when adjusting for changes in their carrier protein levels. By relaxing the confidence of regulated N-glycopeptides (adjusted p < .1), an additional 18 were found to be regulated by >50% compared with their total carrier protein level. In total, 17 of these proteins contained TNF-alpha regulated glycans that had been previously seen to change using PGC-LC-MS/MS analysis of the glycome profile. Interestingly, three of these proteins have previously been associated with GLUT4 storage vesicles (GSVs), including low density lipoprotein receptor-related protein 1 (LRP1), leucyl-cystinyl aminopeptidase (LNPEP), insulin-responsive aminopeptidase (IRAP), and mannose-6-phosphate receptor (M6PR) (41, 42). These vesicles sequester GLUT4 in the cytoplasm and rapidly translocate to the plasma membrane in response to insulin to allow glucose uptake. Aberrant regulation of this process is a key feature of IR, although the precise mechanism for this is incompletely understood. Figure 5C shows the quantification of 95 unique glycopeptides spanning 12 N-glycosylation sites in LRP1. Total protein levels of LRP1 did not change by >1.5-fold with TNF-alpha treatment, while seven glycopeptides were significantly regulated (adjusted p < .1 and ±1.5-fold) by TNF-alpha including an increase in the glycan compositions HexNAcHex5FucNeuAc at Asn-4076. These monosaccharide compositions were identified in the N-glycome profile as comprising glycan structures that display terminal di-galactose epitopes. Figure 5D shows the confident identification of two glycopeptides from LRP1 using HCD and EThcD for sequence identification and glycosylation site localization and CID for confirmation of the glycan structure that had been characterized in the N-glycome profiling. EThcD using a supplemental activation of 15 NCE is dominated by c- and z-type ions but also produces minor amounts of b- and y-type ions and, glycan oxonium ions. The combination of HCD and EThcD is an effective means to identify glycopeptides; however, in our analysis, HCD outperformed EThcD in the identification of glycopeptides (1,563 versus 298 identified) with the median Byonic score higher in HCD compared with EThcD (329 versus 226). As expected, HCD was not able to localize glycosylation sites, while a manual analysis of EThcD spectra was able to localize the glycosylation site on 269 out of 291 glycopeptides identified (Supplemental Table S9 and Supplemental Fig. S2).

DISCUSSION

The systems-wide quantification of the N-glycoproteome with integrated mass spectrometry platforms has revealed distinct levels of regulation of IR in mouse adipocytes. We designed the strategy to differentiate between changes in total protein levels, relative N-glycan occupancy, or changes in N-glycan compositions at specific sites. TNF-alpha-induced IR modulated the abundance of more than 200 proteins. Using available transcriptomics data, ~60 of these were shown to be also regulated at the mRNA level. We quantified 79 unique deamidated peptides on 50 proteins that displayed significantly altered abundance with TNF-alpha treatment. However, when adjusting the regulation of total protein level, only three deamidation sites were significantly changed in abundance. These data suggest there are limited changes in the relative stoichiometry of N-linked glycans. We also normalized the quantification of 56 TNF-alpha regulated N-glycopeptides to the total protein fold-changes. This identified 16 N-glycopeptides that were altered independent of the change in their respective total protein level, suggesting a site-specific change in glycosylation. These data highlight the importance of quantifying these different layers (protein level, occupancy, and glycoform) when assessing glycoprotein regulation. As an example, the deamidated deglycosylated peptide containing Asn-225 from VCAM1 was more than sevenfold increased with TNF-alpha treatment as were the two intact glycopeptides containing Asn-225 with the N-linked glycans, HexNAcHex6FucNeuAc1 and HexNAcHex6Fuc1 NeuAc1. However, the proteome data showed that vascular cell adhesion molecule 1 (VCAM1) total protein levels were similarly sevenfold increased. Hence, when adjusting for the regulation of the expression of this protein, no true occupancy or glycoform changes can be attributed to this VCAM1 glycosylation site.

Glycoproteomics aims to identify and quantify intact glycopeptides in order to elucidate the structure of attached glycans in a site-specific manner on a proteome-wide scale. Mapping the extreme complexity of the mammalian glycoproteome remains challenging, but recent advances in mass spectrometry have dramatically aided this endeavor. While large-scale glycopeptide identification is now feasible and hundreds of glycopeptides have been identified (27, 43–48), systems-wide quantification of the glycoproteome has had
little attention (49). The glycopeptide identification has been advanced by the development of specific glycopeptide search algorithms or modification of existing proteomics pipelines (50–53). While efficient FDR approaches have been adapted to large-scale glycoproteomics, assignment ambiguity is still an issue (54). An inherent limitation of the presented large-scale glycoproteomic analysis is the efficient identification of only glycan compositions as opposed to glycan structures attached to peptides. Correlating the N-glycome profiling structural data with the N-glycoproteomics data can infer site-specific structures but care should be taken if multiple N- or O-linked sites are present on a single peptide. Furthermore, correct monoisotopic peak-picking is important to distinguish glycan compositions, which differ by a few daltons or the presence of other modifications such as acetylation that can confound the inferred assignment (48). We searched the HCD and ETHcD MS/MS data separately while CID MS/MS data were used to support the glycopeptide assignments. Combining data from multiple fragmentation events in a single scoring pipeline has recently been shown to enable confident and automated assignment of glycopeptides (53). Our data show that HCD outperforms ETHcD for glycopeptide identification. However, further comparisons of electron transfer collision-induced dissociation (ETciD) and ETHcD are required, and the reduced identification rate in our data could arise from inefficiencies in the product-ion triggering of ETHcD. Furthermore, we acquired ETHcD high-resolution data in the Orbitrap, and it is likely that optimization and/or comparison to ion-trap detection will increase the identification rates. Finally, increasing charge-density of glycopeptides (e.g. by tandem mass tags (TMT) labeling) combined with improved capacity for ion–ion reactions in the Orbitrap Fusion Lumos are likely to further increase the identification rate of ETHcD.

Previous glycome profiling studies of adipocytes in response to hyperglycemia- or hyperinsulinemia-induced IR have suggested there are no significant changes in N- or O-linked glycan structures on proteins (55, 56). To our knowledge, changes in N-linked glycosylation following inflammatory-induced IR has not been observed in adipocytes. Our N-glycome analysis in mouse adipocytes identified a specific set of regulated glycans in response to TNF-alpha-induced IR. This included the up-regulation of di-galactose-containing N-glycans, which correlated with increased GgtA1 and B4GalT5 expression, and down-regulation of alpha2,3-linked sialic acid, which correlated with decreased ST3Ga6 expression. This correlation was experimentally supported by reducing B4GalT5 expression levels with siRNA, which resulted in decreased amounts of di-galactose-containing glycans. However, it should be noted that, while these experiments support a role for B4GalT5 in the regulation of di-galactose-containing N-glycans, we cannot exclusively say this is a direct regulation. Furthermore, genetic manipulation of GgtA1 is also required to investigate the interplay between alpha- and beta-galactosylation. The role of these glycans and glycosyltransferases in the pathophysiology of IR in adipose tissue is unknown, and further experiments are required to pinpoint the exact mechanisms or consequences of the observed changes. It is also interesting to note that B4GalT5 expression has been implicated in the synthesis of lactosylceramide (57), and aberrant ceramide metabolism has a well-established role in IR (58).

The presented quantitative N-glycoproteomic workflow revealed TNF-alpha regulated glycosylation on specific proteins, including those associated with the specialized insulin-responsive GLUT4 compartment. Coordinated delivery of these GSVs to the plasma membrane to enhance cell surface GLUT4 expression is a hallmark of insulin sensitivity. TNF-alpha treatment specifically regulated high-mannose-containing N-glycans at Asn-200 on IRAP, and LRP1 contained a number of glycosylation sites bearing TNF-alpha regulated N-glycans (Fig. 4C). LRP1 interacts with other GSV proteins, including TBC1D4 and GLUT4 itself, and knock-down of LRP1 has been shown to reduce GLUT4 expression and insulin-stimulated glucose uptake (42). Both IRAP and LRP1 have large luminal domains and have been implicated in the formation of GSVs. Therefore, changes in IRAP and LRP1 glycosylation may modulate their ability to generate GSVs. M6PR has also been reported to be part of GSVs and exhibited altered N-glycans (Fazakerley et al., manuscript in submission). Other adipocyte proteins displaying TNF-alpha altered N-glycans include the lysosomal proteins LAMP1 and LAMP2 and the fatty acid transporter CD36. It has recently been shown that adiposity is associated with increased lysosomal biogenesis and lipid accumulation in adipose macrophages (59). Taken together, it is tantalizing to speculate on a role of subcellular glycosylation, specifically on proteins in lysosomal vesicles and/or GSVs in these cellular processes known to be associated with IR. Furthermore, since adipose tissue has a well-established role as an endocrine organ, additional in vivo investigations of the role of glycosylation on insulin resistance throughout the body is warranted.

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Data Availability: The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium (http://proteomcentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD002435 (username; reviewer47632@ebi.ac.uk, password; 4QvL6JTp).

REFERENCES

1. Rosen, E. D., and Spiegelman, B. M. (2014) What we talk about when we talk about fat. Cell 156, 20–44
2. Abel, E. D., Peroni, O., Kim, J. K., Kim, Y. B., Boss, O., Hadro, E., Minnen, T., Shulman, G. I., and Kahn, B. B. (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 409, 729–733
3. Hotamisligil, G. S., Shargill, N. S., and Spiegelman, B. M. (1993) Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. Science 259, 87–91
4. Mathis, D. (2013) Immunological goings-on in visceral adipose tissue. Cell Metabolism 17, 851–859
5. Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., and Leibel, S. (2003) Obesity is associated with macrophage accumulation in adipose tissue. The J. Clin. Invest. 112, 1796–1808
6. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., Sole, J., Nickolas, A., Rosen, J. S., Tartaglia, L. A., and Chen, H. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J. Clin. Invest. 112, 1821–1830
7. Cawthorn, W. P., and Sethi, J. K. (2008) TNF-alpha and adipocyte biology. FEBS Lett. 582, 117–131
8. Stephens, J. M., and Pekala, P. H. (1991) Transcriptional repression of the GLUT4 factor in 3T3-L1 adipocytes by tumor necrosis factor-alpha. J. Biol. Chem. 266, 21839–21845
9. Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F., and Spiegelman, B. M. (1996) IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science 271, 665–668
10. Stephens, J. M., Lee, J., and Pilch, P. F. (1997) Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J. Biol. Chem. 272, 971–976
11. Ruan, H., Hacohen, N., Golub, T. R., Van Parijs, L., and Lodish, H. F. (2002) Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. Diabetes 51, 1319–1336
12. Bikman, B. T., and Summers, S. A. (2011) Ceramides as modulators of cellular and whole-body metabolism. J. Clin. Invest. 121, 4222–4230
13. Houstis, N., Rosen, E. D., and Landers, E. S. (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440, 944–948
14. Hoehn, K. L., Salmon, A. B., Hohnen-Behrens, C., Turner, N., Hoy, A. J., Maghazli, G. J., Stocker, R., Van Remmen, H., Kraegen, E. W., Cooney, G. J., Richardson, A. R., and James, D. E. (2009) Insulin resistance is a cellular antioxidant defense mechanism. Proc. Natl. Acad. Sci. U.S.A. 106, 17787–17792
15. Banno, T., Gazel, A., and Blumenberg, M. (2004) Effects of tumor necrosis factor-alpha (TNF alpha) in epidermal keratinocytes revealed using global transcriptional profiling. J. Biol. Chem. 279, 32633–32642
16. Ohtsubo, K., and Mart, J. D. (2011) Pathway to diabetes through attenuation of pancreatic beta cell glycosylation and glucose transport. Nature Medicine 17, 1067–1075
17. Ohtsubo, K., Takamatsu, S., Minowa, M. T., Yoshida, A., Takeuchi, M., and Mart, J. D. (2005) Dietary and genetic control of glucose transport 2 glucose transport promoter stimulates insulin secretion in suppressing diabetes. Cell 123, 1307–1312
18. Shewan, A. M., Marsh, B. J., Melvin, D. R., Martin, S., Gould, G. W., and James, D. E. (2000) The cytosolic C-terminus of the glucose transporter GLUT4 contains an acidic cluster endosomal targeting motif distal to the dileucine signal. Biochem. J. 350, 99–107
19. Ong, S. E., and Mann, M. (2006) A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). Nature Protocols 1, 2650–2660
20. Myaling, S., Palmisano, G., Hofjrup, P., and Thaysen-Andersen, M. (2010) Utilizing ion-pairing hydrophilic interaction chromatography solid phase extraction for efficient glycopeptide enrichment in glycoproteomics. Anal. Chem. 82, 5598–5609
21. Palmisano, G., Lendal, S. E., Engholm-Keller, K., Leth-Larsen, R., Parker, B. L., and Larsen, M. R. (2010) Selective enrichment of sialic acid-containing glycopeptides using titanium dioxide chromatography with analysis by HILIC and mass spectrometry. Nature Protocols 5, 1974–1982
22. Parker, B. L., Thaysen-Andersen, M., Solis, N., Scott, N. E., Larsen, M. R., Graham, M. E., Packer, N. H., and Cordwell, S. J. (2013) Site-specific glycan-peptide analysis for determination of N-glycoproteome heterogeneity. J. Proteome Res. 12, 5791–5800
23. Fresen, G. K., Atebaar, A. F., van den Toorn, H., Notling, D., Grieb-Ramming, J., Heck, A. J., and Mohammed, S. (2012) Toward full peptide sequence coverage by dual fragmentation combining electron-transfer and higher-energy collision dissociation tandem mass spectrometry. Anal. Chem. 84, 9668–9673
24. Saba, J., Dutta, S., Hemenway, E., and Viner, R. (2012) Increasing the productivity of glycopeptides analysis by using higher-energy collision dissociation-accurate mass-product-dependent electron transfer dissociation. Int. J. Proteomics 2012, 560391
25. Wu, S. W., Pu, T. H., Viner, R., and Khoo, K. H. (2014) Novel LC-MS(2) product dependent parallel data acquisition function and data analysis workflow for sequencing and identification of intact glycopeptides. Anal. Chem. 86, 5478–5486
26. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnol. 26, 1367–1372
27. Käll, L., Canterbury, J. D., Weston, J., Noble, W. S., and MacCoss, M. J. (2007) Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat. Methods 4, 923–925
28. Bern, M., Kii, Y. J., and Becker, C. (2012) Byonic: Advanced peptide and protein identification software. In Current Protocols in Bioinformatics, A. D. Baxevanis, Alex Bateman, Sorin Draghici, William R. Pearson, and Lincoln D. Stein. eds., Chapter 13, Unit13 20
29. Jensen, P. H., Karlsson, N. G., Kolarich, D., and Packer, N. H. (2012) Structural analysis of N- and O-glycans released from glycoproteins. Nature Protocols 7, 1299–1310
30. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408
31. Wright, H. L., Thomas, H. B., Moots, R. J., and Edwards, S. W. (2013) RNA-seq reveals activation of both common and cytokine-specific path-
Insulin Resistance Alters the Protein Glycosylation of Adipocytes

38. Qi, L., Saberi, M., Zmuda, E., Wang, Y., Altarejos, J., Zhang, X., Dentin, R., Hedrick, S., Bandyopadhyay, G., Hai, T., Olefsky, J., and Montminy, M. (2009) Adipocyte CREB promotes insulin resistance in obesity. Cell Metabolism 9, 277–286

39. Wright, H. T. (1991) Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. Crit. Rev. Biochem. Mol. Biol. 26, 1–52

40. Abrahams, J. L., Packer, N. H., and Campbell, M. P. (2015) Relative quantification of multi-antennary N-glycan classes: combining PGC-LC-ESI-MS with exoglycosidase digestion. Analyst 140, 5444–5449

41. Larance, M., Ramm, G., Stöckli, J., van Dam, E. M., Winata, S., Wasinger, V., Simpson, F., Graham, M., Junutula, J. R., Guilhaus, M., and James, D. E. (2005) Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking. J. Biol. Chem. 280, 37803–37813

42. Jedrychowski, M. P., Gartner, C. A., Gygi, S. P., Zhou, L., Herz, J., Kandror, K. V., and Pilch, P. F. (2010) Proteomic analysis of GLUT4 storage vesicles reveals LRPI to be an important vesicle component and target of insulin signaling. J. Biol. Chem. 285, 104–114

43. Harazono, A., Kawasaki, N., Itoh, S., Hashii, N., Matsuishi-Nakajima, Y., Kawanishi, T., and Yamaguchi, T. (2008) Simultaneous glycosylation analysis of human serum glycoproteins by high-performance liquid chromatography/tandem mass spectrometry. J. Chromatogr. 869, 20–30

44. Kuroguchi, M., Matsushita, T., Amano, M., Furukawa, J., Shinohara, Y., Sun, S., Hoti, N., Chen, L., Yang, S., Pasay, J., Rubin, A., and Zhang, H. (2015) Integrated proteomic and glycoproteomic analyses of prostate cancer cells reveal glycoprotein alteration in protein abundance and glycosylation. Mol. Cell. Proteomics 14, 2753–2763

45. Mayampurath, A. M., Wu, Y., Segue, Z. M., Mechref, Y., and Tang, H. (2011) Improving confidence in detection and characterization of protein N-glycosylation sites and microheterogeneity. Rapid Commun. Mass Spectr. 25, 2007–2019

46. Woodin, C. L., Hua, D., Maxon, M., Rebecchi, K. R., Go, E. P., and Desaire, H. (2012) GlycoPep grader: A web-based utility for assigning the composition of N-linked glycopeptides. Anal. Chem. 84, 4821–4829

47. Wu, S. W., Liang, S. Y., Pu, T. H., Chang, F. Y., and Khoo, K. H. (2013) Sweet-Heart - an integrated suite of enabling computational tools for automated MS2/MS3 sequencing and identification of glycopeptides. J. Proteomics 84, 1–16

48. Mayampurath, A., Yu, C. Y., Song, E., Balan, J., Mechref, Y., and Tang, H. (2014) Computational framework for identification of intact glycopeptides in complex samples. Anal. Chem. 86, 453–463

49. Medzihradszky, K. F. (2014) Noncovalent dimer formation in liquid chromatography-mass spectrometry analysis. Analyst 139, 8906–8909

50. Parry, S., Hadaschik, D., Blacher, C., Kumaran, M. K., Bochkina, N., Morris, H. R., Richardson, S., Altman, T. J., Gauguer, D., Siddle, K., Scott, J., and Dell, A. (2006) Glycomics investigation into insulin action. Biochim. Biophys. Acta 1760, 652–668

51. Lim, J. M., Wollaston-Hayden, E. E., Teo, C. F., Hausman, D., and Wells, L. (2014) Quantitative secretome and glycome of primary human adipocytes during insulin resistance. Clinical Proteomics 11, 20

52. Kumagai, T., Sato, T., Natsuka, S., Kobayashi, Y., Zhou, D., Shinkai, T., Hayakawa, S., and Furukawa, K. (2010) Involvement of murine beta-1,4-galactosyltransferase V in lactosylceramide biosynthesis. Glycoconj. J. 27, 885–895

53. Chavez, J. A., and Summers, S. A. (2012) A ceramide-centric view of insulin resistance. Cell Metabolism 15, 585–594

54. Medina, D. B., and Hetz, C. (2013) Proteostasis impairment: At the intersection between Alzheimer’s disease and diabetes. Cell Metabolism 18, 771–772