



# Adiporedoxin, an upstream regulator of ER oxidative folding and protein secretion in adipocytes

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## ABSTRACT

**Objective:** Adipocytes are robust protein secretors, most notably of adipokines, hormone-like polypeptides, which act in an endocrine and paracrine fashion to affect numerous physiological processes such as energy balance and insulin sensitivity. To understand how such proteins are assembled for secretion we describe the function of a novel endoplasmic reticulum oxidoreductase, adiporedoxin (Adrx).

**Methods:** Adrx knockdown and overexpressing 3T3-L1 murine adipocyte cell lines and a knockout mouse model were used to assess the influence of Adrx on secreted proteins as well as the redox state of ER resident chaperones. The metabolic phenotypes of *Adrx* null mice were characterized and compared to WT mice. The correlation of Adrx levels BMI, adiponectin levels, and other inflammatory markers from adipose tissue of human subjects was also studied.

**Results:** Adiporedoxin functions *via* a CXXC active site, and is upstream of protein disulfide isomerase whose direct function is disulfide bond formation, and ultimately protein secretion. Over and under expression of *Adrx in vitro* enhances and reduces, respectively, the secretion of the disulfide-bonded proteins including adiponectin and collagen isoforms. On a chow diet, *Adrx* null mice have normal body weights, and glucose tolerance, are moderately hyperinsulinemic, have reduced levels of circulating adiponectin and are virtually free of adipocyte fibrosis resulting in a complex phenotype tending towards insulin resistance. Adrx protein levels in human adipose tissue correlate positively with adiponectin levels and negatively with the inflammatory marker phospho-Jun kinase.

**Conclusion:** These data support the notion that Adrx plays a critical role in adipocyte biology and in the regulation of mouse and human metabolism *via* its modulation of adipocyte protein secretion.

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**Keywords** Adipocyte; Adipokine; Protein secretion; Endoplasmic reticulum; Oxidoreductase; Disulfide bond formation

## 1. INTRODUCTION

In addition to fat storage and release, white adipose tissue (WAT) makes and secretes adipokines, hormone-like polypeptides, which act in an endocrine and paracrine fashion to affect numerous physiological processes such as energy balance and insulin sensitivity [1,2]. In addition, fat cells in adipose tissue are maintained as an organ by extracellular matrix proteins including multiple collagen isoforms whose cellular origin is not completely clear [3]. The initial folding and assembly of all secreted and integral membrane proteins, takes place in the endoplasmic reticulum (ER) and is mediated by the actions of numerous enzymes and chaperones [4–6]. About 1/3 of all cellular proteins pass through the ER and the vast majority of these have inter- and/or intra-molecular disulfide bonds, the formation of which also takes place in the ER mediated by oxidoreductases of the thioredoxin superfamily [6–8]. Improper disulfide bond formation leads to protein misfolding and, potentially, ER stress, which can

have deleterious consequences for cells, for example, apoptosis in the endocrine pancreas due to insulin misfolding [9,10]. Therefore, while normal ER function is essential in all cells, it is especially important in secretory cells such as adipocytes and pancreatic beta cells.

Adiponectin assembly and disulfide bond formation impose a particular burden on the adipocyte ER because it circulates at high concentrations, ca. 30 nM, yet is turned over quite rapidly in blood [11]. Adiponectin has a complicated tertiary structure with post-translational modifications that include the ER-dependent formation of intermolecular disulfide bonds that are required for oligomerization into its most physiologically relevant form, an octadecamer (the high molecular weight or HMW form) [12–14], as well as hydroxylation, glycosylation and proline isomerization all of which occur in its collagenous domain [15–17]. Adiponectin must therefore be properly and efficiently assembled and secreted at high rates, and malfunctions of this process will lead to ER stress in the adipocyte and subsequent

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perturbation of fat cell and organismal metabolism such as insulin resistance and type 2 diabetes [18]. The assembly and secretion of collagens from adipocytes (and other cells) also requires disulfide bonds [17], and excess collagen deposition leads to a fibrotic pathology in adipose tissue [19].

What little is known about disulfide bond formation in the adipocyte ER involves adiponectin assembly, which requires the action of the enzyme endoplasmic reticulum oxidoreductase 1 $\alpha$  (ERo1 $\alpha$ ), a primary electron acceptor that uses molecular oxygen and a flavin nucleotide to initiate oxidative protein folding [20]. ERo1 $\alpha$  does not interact directly with substrates such as adipokines to mediate S–S bond formation, rather it oxidizes members of the protein disulfide isomerase (PDI) family, which interact with substrates and mediate correct disulfide bond formation and protein folding [20–22]. ERo1 $\alpha$  has also been shown to interact with endoplasmic reticulum protein, ERp44, to mediate what has been called dynamic ER retention of substrates [23]. The combined actions of ERo1 $\alpha$  and ERp44 have been shown to play a role in adiponectin assembly and secretion [24,25]. A third PDI relative implicated in adiponectin multimerization is the disulfide bond A oxidoreductase-like protein (DsbA-L) [26]. Unlike ERo1 $\alpha$  and ERp44, it lacks both active site cysteines required for redox function and a classical ER localization signal, but it may instead have a chaperone function in protein folding. None of the above proteins has a notable tissue-specific distribution although the expression of DsbA-L is somewhat higher in adipose than in other tissues examined, and it is induced upon differentiation of 3T3-L1 cells [26].

We performed a proteomic analysis of GLUT4 storage vesicles (GSVs) that were selectively immunoabsorbed with anti-GLUT4-coupled beads following depletion of GLUT4-precursor compartments [27]. Amongst the proteins we discovered in the GSV-precursor membranes were several potential ER residents including one that was known at the time only as a possible secreted protein (c10orf58) whose mRNA was expressed in human testes [28]. Its sequence revealed a CXXC motif, which is the active site sequence of an oxidoreductase [6,21]. We determined by qPCR that its mRNA was expressed in mouse testes, but we also saw that mRNA expression in brown and white fat was an order of magnitude higher than in any other mouse tissue except testes (data not shown), which we confirm here for protein expression (Figure 1). Consequently, we are calling this molecule adiporedoxin (Adrx), and we show here that it plays an important role in the assembly and secretion of disulfide-bond containing proteins, in particular, adiponectin in murine and human cells and adiponectin and collagens in murine cells. Its absence in mice leads to a complex and mixed phenotype of lower adipokine secretion and lower adipocyte collagen deposition along with adipocyte ER stress and hyperinsulinemia.

## 2. MATERIALS AND METHODS

### 2.1. Animals

Primary adipocytes from male Sprague–Dawley rats (175–200 g, Harlan Laboratories, Indianapolis, IN) were isolated and fractionated as described [27]. The same fractionation protocol was performed for 3T3-L1 cultured murine adipocytes. Age matched, 8 week old C7BL/6 male and female mice were purchased from Jackson Laboratory (Farmington, CT), maintained at 22 °C with a light–dark cycle (light from 0700 to 1900h), and allowed free access to food and water. At 10 weeks of age, mice were body weight matched into groups fed either HF (45% of calories, mainly as lard) or LF (15%) diets, then sacrificed as described [29]. All females were euthanized at the pro-estrus phase. For Adrx tissue distribution, mice were

perfused with PBS, tissues were carefully dissected with particular attention to removing any fat, immediately mechanically homogenized in a tissue grinder with RIPA buffer containing a protease inhibitor cocktail and subjected to a 1000  $\times$  g spin to remove debris. The supernatant was collected for SDS-PAGE and Western blot analysis. For fat tissue, the infranatant below the fat cake was collected for analysis.

The *Adrx* knockout mice were generated by microinjecting targeted ES cells (KOMP, derived from C57BL/6N, VG15730, clone 15730A-H5) into C57BL/6N blastocysts, and these gave rise to male chimeras with significant ES cell contribution (as determined by coat color). By mating with C57BL/6N females and genotyping the offspring by PCR analysis, germ line transmission was confirmed. Male and female heterozygous F1 animals were interbred to obtain *Adrx* knockout (*Adrx* KO) animals. Male animals only were analyzed at >8 weeks of age. The animals were maintained in a pathogen-free animal facility at 22 °C under a 12-h light/12-h dark cycle with access to a standard rodent chow. Except when specifically noted, all mice used for *in vivo* or *in vitro* studies were fasted for 4–6 h starting from early morning prior to use. For tissue harvesting, mice were sacrificed under CO<sub>2</sub> anesthesia, and tissues were rapidly taken and immediately frozen in liquid nitrogen and stored at –80 °C until biochemical analysis. Tissue and cell secreted collagen content was measured using Sirius Red Total Collagen Detection Kit (Chondrex, Redmond, WA, #9062). Mouse serum total and HMW adiponectins were measured by using ELISA kit from ALPCO (Salem, NH, #47-ADPMS-E01).

### 2.2. Primary human adipose cells

Human adipose stromal vascular cells (SVC) were isolated from adipose tissue by collagenase digestion, grown and differentiated as previously described [30]. Adipocytes (day 10 post differentiation) were transfected with control or a combination of two *Adrx*-directed siRNA (SI04213587 and SI04959066, Qiagen, Hilden, Germany) using Lipofectamine and PLUS reagents (Life Technologies, Carlsbad, CA).

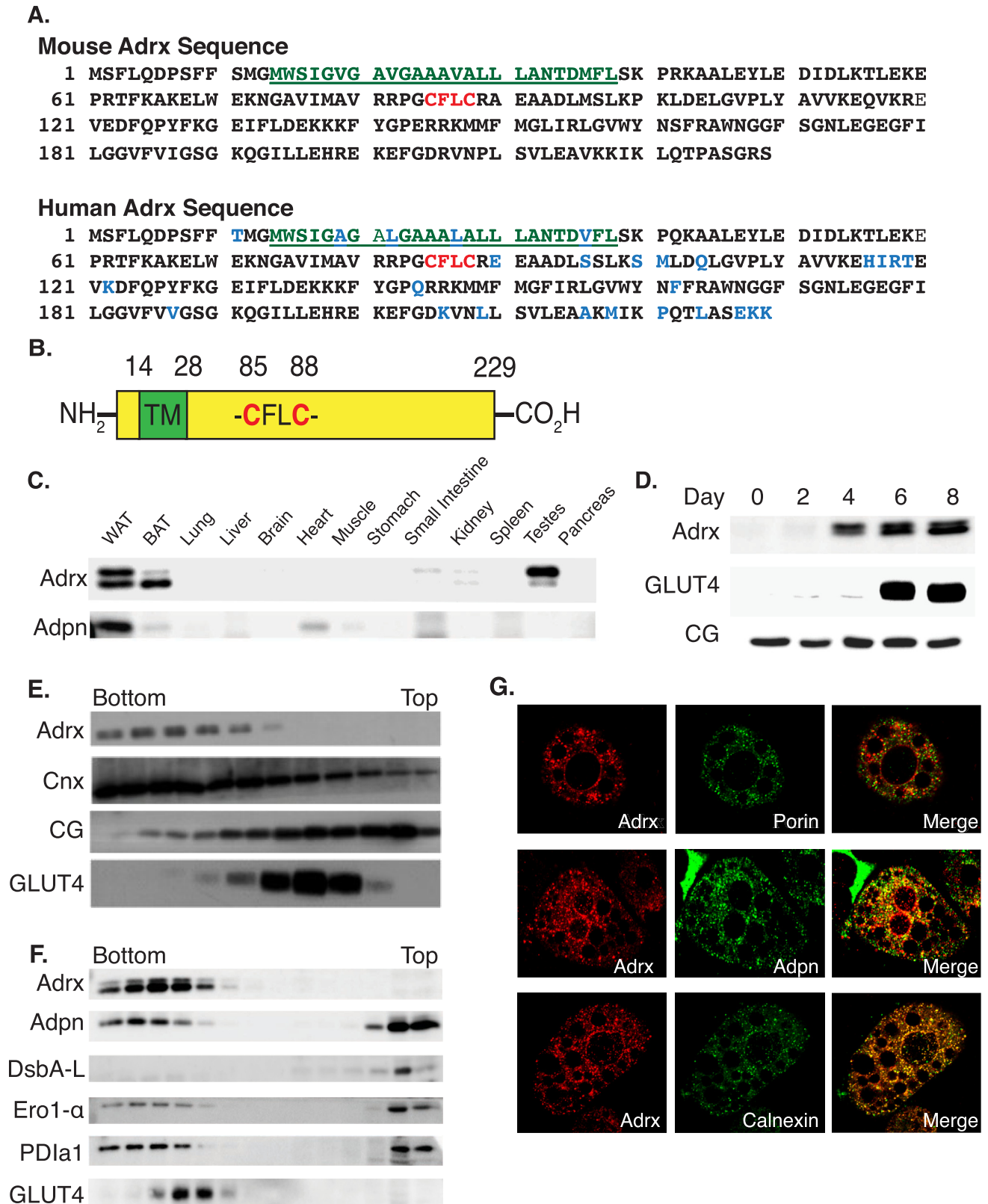
### 2.3. Cell culture

Murine 3T3-L1 cells were cultured, differentiated, and maintained as described previously [27]. Human embryonic kidney (HEK)-293T cells were cultured in DMEM with 10% FBS. For *Adrx* overexpression, confluent 3T3-L1 fibroblasts were infected with a pBABE retrovirus construct driving *Adrx* cDNA using the following oligo primers: sense sequence containing BamHI restriction site, 5'-tttggatccatgctctttctc-caggac-3' and an antisense sequence containing an EcoRI restriction site, 5'tttgaaattctcagcttctcccggacgctggagt-3', then differentiated for subsequent analysis as fat cells. Site directed mutagenesis of *Adrx* was performed using a Quickchange Mutagenesis Kit (Agilent, Santa Clara, CA) following the manufacturer's instructions.

For *Adrx* silencing, mouse lentiviral vectors driving several shRNA sequences were purchased from Open Biosystems (Huntsville, AL) and transfected into HEK-293T cell with trans-IT 293 (Mirus, Madison, WI) according to the manufacturer's instructions. Supernatant containing retrovirus was harvested 48 h later and used to infect confluent 3T3-L1 fibroblasts prior to their differentiation. Four pLKO.1 c10orf58 mouse lentiviral vectors were tested. All sequences were effective with target # 3 showing the best knockdown efficiency (>90%), and it was used for all of the adiporedoxin studies.

### 2.4. qPCR

Total RNA was isolated, quantified and reverse-transcribed using a commercial cDNA synthesis kit. Quantitative PCR (qPCR) was performed with commercially available TaqMan probes (Thermo Fisher



**Figure 1:** Adiporedoxin (Adrx) is an endoplasmic reticulum targeted peroxiredoxin-related protein enriched in adipocytes. **A.** The sequences and domains **B.** of mouse and human adiporedoxin are shown, highlighting the transmembrane region and the active site cysteines **C.** The tissue distribution of adiporedoxin and adiponectin (Adpn) is shown for equal protein amounts of cell lysates subjected to SDS-PAGE and Western blotting detection. **D.** Adrx protein expression is induced during 3T3-L1 cell differentiation from fibroblasts (day 0) to mature fat cells (day 8). An equal protein amount of cell lysates from the days indicated was subjected to SDS-PAGE and immunoblotted for the selected proteins. **E.** The internal membrane fraction (0.5 mg protein) from isolated primary adipocytes was subjected to sucrose gradient centrifugation; odd-numbered fractions were collected and blotted

Scientific) or SYBR primers. Primer sequences will be available upon request.

## 2.5. Gel electrophoresis and immunoblotting

Proteins were resolved by SDS-PAGE as described [31]. Non-denaturing PAGE separation of adiponectin oligomers was performed as described [13]. Gels were transferred to polyvinylidene difluoride (Biorad, Hercules, CA) membranes pretreated with methanol in 25 mM Tris, 192 mM glycine. Membranes were blocked with 10% nonfat dry milk in PBS containing 0.1% Tween 20 for 1 h at room temperature. Membranes were then probed with the primary antibodies for either overnight at 4 °C or 2 h at room temperature and incubated with Horseradish peroxidase-conjugated secondary antibodies (Sigma—Aldrich, St. Louis, MO). Signals were enhanced with chemiluminescence reagents (Perkin Elmer Life Sciences, Boston, MA) for detection of Western signals using a Fujifilm LAS-4000 scanner or Autoradiography Film (Molecular Technologies, St. Louis, MO). Antibodies to the indicated proteins were from the following sources: GLUT4, cellugyrin, IRAP and Adrx (21st Century Biochemical, Hopkinton, MA); adipisin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); ER $\alpha$  (Abnova, Taipei, Taiwan); adiponectin (Affinity Bioreagents, Golden, CO);  $\beta$ -actin, tubulin (Sigma—Aldrich, St. Louis, MO); DsbA-L and calnexin (Abcam, Cambridge, MA). Antibodies to the following were generous gifts: aP2 (Dr. David Bernlohr, University of Minnesota), insulin receptor (Dr. Jongsoo Lee, Joslin Diabetes Center).

## 2.6. Immunofluorescence

On Day 6 of differentiation, cells were trypsinized and plated onto coverslips in 6 well plates. On day 8 of differentiation cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.1% saponin (Sigma—Aldrich, St. Louis, MO), 0.4% BSA in PBS (solution A) for 10 min and then blocked for 1 h at room temperature in 5% normal goat serum (Sigma—Aldrich, St. Louis, MO) in PBS. Primary antibodies were incubated with fixed cells overnight at 4 °C at a dilution of 1/100 in 1% normal goat serum in PBS. After staining cells were washed four times with solution A and incubated with Alexa-Fluor 488 anti-mouse or Alexa-Fluor 555 anti-rabbit (Life Technologies, Carlsbad, CA) at a dilution of 1/1000 in 1% normal goat serum in PBS for 1 h at room temperature. The cells were washed again for 3 times with solution A and then mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The stained cells were observed using a Nikon deconvolution wide-field Epifluorescence system and images were processed using NIH ImageJ software (<http://imagej.nih.gov/ij/>).

## 2.7. Mass spectrometry

### 2.7.1. Sample preparation and peptide TMT-labeling

Cultured adipocytes were lysed with a mechanical homogenizer, disulfide bonds were reduced with DTT, and cysteine residues alkylated with iodoacetamide essentially as previously described [32]. Isobaric labeling of the peptides was accomplished with six-plex tandem mass tag (TMT) reagents (Thermo Scientific, Rockford, IL). Reagents, 0.8 mg, were dissolved in 40  $\mu$ l acetonitrile (ACN) and 15  $\mu$ l of the solution were added to 100  $\mu$ g of peptides dissolved in

100  $\mu$ l of 50 mM HEPES, pH 8.5 After 1 h at room temperature, the reaction was quenched by adding 8  $\mu$ l of 5% hydroxylamine. Peptides were labeled with all six reagents (126–131), combined and subjected to C18 SPE.

### 2.7.2. Liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS)

All LC-MS/MS experiments were performed on an LTQ Orbitrap Velos (Thermo Scientific, Rockford, IL) equipped with a Famos autosampler (LC Packings, Sunnyvale, CA) and an Agilent 1100 binary HPLC pump (Agilent Technologies, Santa Clara, CA) as described [33].

### 2.7.3. Data processing: MS2 spectra assignment, data filtering and quantitative data analysis

Assignment of MS2 spectra was performed using the Sequest algorithm by searching the data against a protein sequence database containing all known translated proteins from the mouse IPI database (version 3.60), and known contaminants such as porcine trypsin, and human keratin. This forward (target) database component was followed by a decoy component including all listed protein sequences in reversed order.

For quantification, the total signal intensity across all peptides quantified was summed for each TMT channel, and all intensity values were normalized to account for potentially uneven TMT labeling. The intensities for all peptides of a given protein were summed to derive an overall protein abundance value for each TMT signal. Hierarchical clustering using Pearson's correlational analysis was conducting using MultiExperiment Viewer (TM4 Microarray Software Suite) and Principle component analysis based on correlations was performed using JMP statistical software (version 10 Pro).

Protein ratios across Adrx cell lines between KD to control and OE to control were calculated based on their normalized TMT ratios. Principle component (PCA) analysis, GO classifications and enrichment calculations were performed using DAVID online bioinformatics tools. The resulting clusters are plotted for GO Cellular Components, GO Biological Processes and GO Molecular Function annotations. Representative proteins are highlighted with their corresponding ratios.

## 2.8. Pegylation assay

3T3-L1 cultured adipocytes at day 8 of differentiation were treated with 5 mM dithiothreitol (DTT) for 5 min, 3 mM diamide for 5 min, or various concentrations of H<sub>2</sub>O<sub>2</sub> for 10 min. Cells were denatured and precipitated in ice-cold PBS containing 20% trichloroacetic acid (TCA), incubated on ice for 1 h, and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was discarded and the pellet was washed 3 times with ice-cold acetone, dried, and resuspended in reaction buffer (80 mM Tris-HCl, pH 6.8, and 2% SDS) containing 5 mM methoxy-polyethylene glycol (average molecular weight, 2000)-maleimide (MalPEG-2K) (NOF Corporation, White Plains, NY) [34]. Samples were left in the dark for 60 min at room temperature. The alkylation reaction was stopped by boiling for 5 min after the addition of an equal volume of 4X Laemmli loading buffer.

## 2.9. Study approval

The animal studies were approved by the Institutional Animal Care and Use Committee and the human fat cells were obtained from subjects

for the proteins indicated, adiporedoxin (Adrx), calnexin (Cnx), cellugyrin (CG) and GLUT4. F. 3T3-L1 cells were lysed, processed and subjected to sucrose gradient analysis as for the primary adipocytes of 1e. Additional proteins analyzed are adiponectin (Adpn), disulfide bond A oxidoreductase-like (DsbA-L), endoplasmic reticulum oxidoreductase 1-alpha (ER $\alpha$ ). G. Immunofluorescence of differentiated 3T3-L1 cells comparing the localization of Adrx with calnexin, adiponectin (Adpn) and porin. The data for each part of this figure are representative of three or more independent experiments.

who gave informed consent as approved by Institutional Review Boards of Boston University School of Medicine.

### 3. RESULTS

#### 3.1. Adiporedoxin (Adrx) is an ER protein highly expressed in white (WAT) and brown (BAT) adipose tissue and is induced during adipocyte differentiation

Figure 1A shows the sequences of mouse and human Adrx with their –CXXC– active sites (red) and transmembrane (TM) region/leader sequences (green underlined), both domains indicated in the cartoon of Figure 1B. Figure 1C and D show respectively, the tissue distribution of Adrx protein in mice and the time-dependency of its expression as a function of 3T3-L1 adipocyte differentiation. As expected, Adrx protein expression was relatively high in testis, and although great care was taken in removing fat from the other tissues, traces of Adrx (compared to fat and testes) can be observed in small intestine and spleen, which probably reflects low level expression in those tissues (Figure 1C). Like many proteins that are highly expressed in adipocytes, GLUT4 for example [35], Adrx was markedly induced as preadipocytes differentiate (Figure 1D). Like the murine cells, cultured human adipocytes express Adrx mRNA and protein in a differentiation dependent fashion and its knockdown in these cells results in diminished adiponectin secretion (Figure S1). Adrx mRNA is also expressed in human adipose tissue to a much greater degree than in other human organs (Figure S1).

We determined by sucrose gradient fractionation in primary rat adipocytes (Figure 1E) that Adrx was colocalized with the ER membrane marker, calnexin (Cnx) [36], and was well separated from GLUT4-rich vesicles. Its partial co-localization with cellugyrin (CG), a marker for diverse intracellular vesicles [37], accounts for its appearance in GSV precursor membranes that allowed its initial identification [27]. To put Adrx into the context of other possible ER contributors to fat cell ER function, disulfide bond formation and protein secretion, we subjected membranes from 3T3-L1 adipocytes to sucrose gradients and showed that Adrx overlaps significantly with proteins of the ER lumen, namely adiponectin (Adpn), ER $\alpha$  and PD1a1 (Figure 1F). These last three proteins are soluble and were partially released from the ER during cell fractionation and therefore migrated at the top of the gradient, whereas Adrx did not, confirming its integral membrane association. On the other hand, DsbA-L distributed only to the top of the gradient, indicating that it is unlikely to be a resident of the ER lumen. Lastly, we confirmed by immunofluorescence microscopy in 3T3-L1 adipocytes, the nearly complete co-localization of Adrx with calnexin, its partial localization with adiponectin and its minimal, if any, co-localization with porin [38], hence mitochondria (Figure 1G).

#### 3.2. Adrx levels affect adiponectin and collagen secretion

Given the CXXC motif of adiporedoxin and its ER localization, its role in disulfide bond formation and subsequent protein secretion seemed likely. Thus we generated Adrx knockdown and overexpressing 3T3-L1 cells (Figure 2A) and monitored secreted (Figure 2B,C) adiponectin and collagen (Figure 2D) comparing them to control (scrambled shRNA). As previously noted, adiponectin is found circulating in what are often termed low, medium and high molecular weight oligomers corresponding to trimer, the first formed disulfide-linked oligomer (LMW), hexamer (MMW) and larger oligomers (HMW) [14]. As shown in Figure 2B in a non-denaturing, non-reducing gel for 4 h of secretion, all Adpn multimers were affected to the same extent by alterations in Adrx expression. That is, secretion is inhibited in the knockdown cells and enhanced in the over expressing cells as compared to controls and this

is shown quantitatively in Figure 2C. As noted in the Introduction, adiponectin and collagen have some similar features, disulfide bonds, a triple helical region and post-translational modifications, and we also determined the effects of altered Adrx expression on collagen secretion. It is inhibited or enhanced, respectively, by Adrx knockdown and over expression (Figure 2D). Moreover, and like cultured fat murine cells (Figure 2B), siRNA mediated knockdown of Adrx in primary human adipocyte cultures inhibited the secretion of all adiponectin oligomers (Supplemental Figure 1). Thus, these aspects of Adrx biochemistry *in vitro* appeared identical in mouse and human fat cells. If the overall effect of Adrx expression levels results in altered assembly of mature proteins, hence altered secretion, we would expect that cell-associated adiponectin would be enhanced by its knockdown and decreased by its over expression and this is exactly what we observe (Figure 2A). In addition, the insulin receptor which has numerous intra- and inter-molecular disulfide bonds [39] also shows altered expression due to Adrx manipulation, as expected, less receptor in the knockdown and more in the over expressers (Figure 2E). In this case, it was the steady state levels of an integral membrane protein that were being altered.

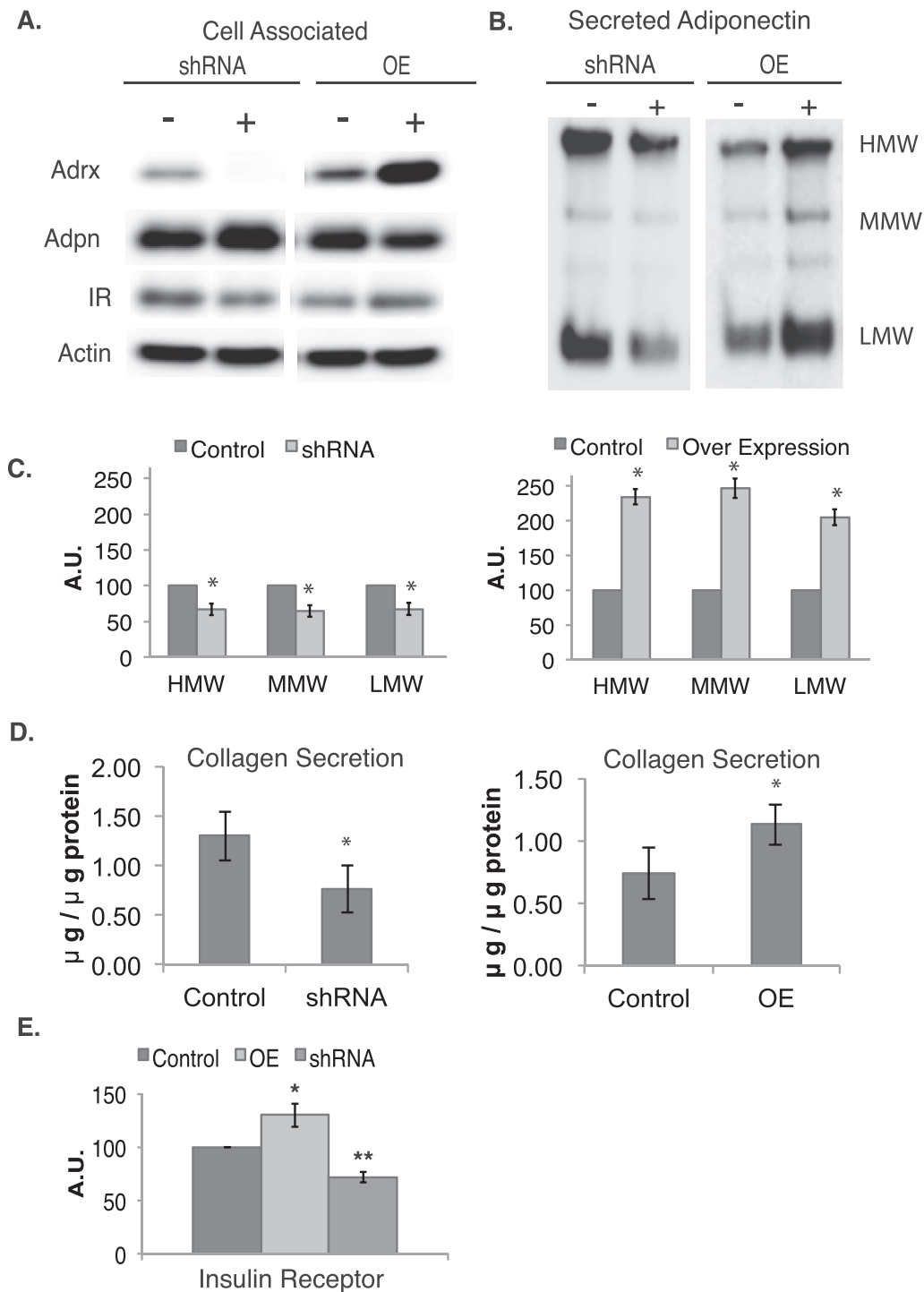
#### 3.3. Adiporedoxin is a redox-regulated protein, which catalyzes the oxidation of ER chaperones

To directly confirm that Adrx functioned as a redox-regulated protein, we used N-ethylmaleimide PEGylation [40] to assay for changes in the adipocyte ER oxidation state as reflected by mobility shifts in Adrx and PD1a1. This reagent covalently reacts with reduced cysteines and retards protein mobility by 2K when run in non-reducing SDS-PAGE. As shown in Figure 3A, control cells exhibited a mixture of oxidized and reduced PDI and Adrx, and cells treated with the strong oxidizing reagent diamide [41] showed fully oxidized proteins and cells treated with the reducing agent, dithiothreitol (DTT), showed reduced mobility due to PEGylation. As a control, a mutant Adrx (SFLS) lacking the two active site cysteines did not show this shift (Figure 3A, right hand panel).

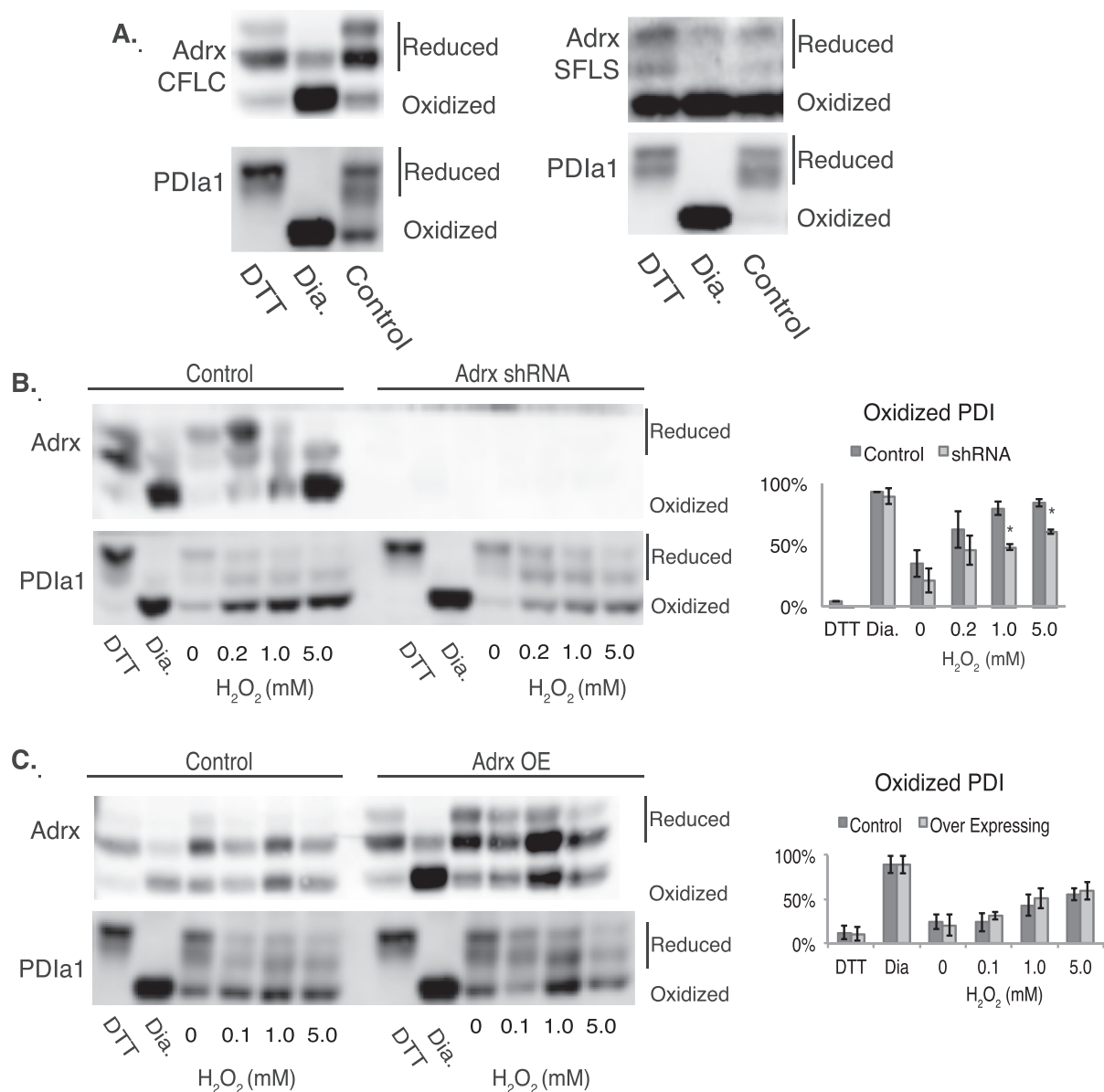
To determine if Adrx could affect protein secretion through re-oxidation of chaperone proteins, we determined the redox state of PD1a1 in cells over and under expressing adiporedoxin (Figure 3B, C). Knocking down Adrx decreased the oxidized form of PD1a1 in the basal state and in response to all doses of hydrogen peroxide (Figure 3B). Overexpression of Adrx caused a slight but statistically insignificant increase in the oxidized forms of PD1a1 (Figure 3C) consistent with the enhanced secretion under these conditions.

#### 3.4. Adrx expression levels affect the adipocyte proteome

We used Tandem Mass Tag technology (TMT) and quantitative mass spectrometry [42,43] to interrogate the proteome of control 3T3-L1 adipocytes (CON) and Adrx overexpressing (OE) and knockdown (KD) adipocytes. Overall, we were able to identify ca. 4500 proteins at a 1% protein false discovery rate (Supplemental Table S1). Principal component analysis (Figure 4A) of the proteomic data shows good agreement between the biological replicates, and we observed that the majority of the proteome was unaffected by Adrx expression. Figure 4B shows a heat map of all proteins quantified and Gene ontology (GO) enrichment analysis for proteins in the overexpressing cells showed a significant increase in cell associated oxidoreductase and thioredoxin-related proteins and decreases in secreted adiponectin and collagen (Figure 4C), the latter two as would be expected from the data of Figure 2. Thus the amount of cell-associated, disulfide-bonded adipokines, adiponectin (Adpn) and resistin (Rtn) [44,45] was less than in controls in the Adrx overexpressing cells and increased (cell retention)



**Figure 2:** Adiporedoxin levels affect the secretion of adiponectin (Adpn) and collagen, and the insulin receptor (IR) levels. Day 8 differentiated murine adipocytes (3T3-L1) infected with Adrx shRNA, WT Adrx cDNA (OE) or a scrambled sequence (–) were washed 3X with serum free medium, and then incubated for 4 h (A–D). Cell lysates and supernatants were harvested, subjected to SDS-PAGE and Western blotted for the indicated proteins. **A.** Shown is a representative experiment for Adrx knockdown and over expressing cells where lysates were prepared and subjected to SDS-PAGE under reducing conditions showing the total cellular levels of Adrx, Adpn and the insulin receptor (IR), actin as the loading control. **B.** Cell supernatants from a representative experiment were collected from control (–), knockdown (shRNA, +) and Adrx overexpressing cells (OE, +) after 4 h and subjected to non-denaturing, non-reducing gel electrophoresis, the forms as indicated (HMW, MMW and LMW). **C.** The results of scanned gels for the 3 forms shown in **B** are given as the S.E.M.,  $n = 3$ ,  $p < 0.001$  for the over expressers and  $n = 9$ ,  $p < 0.002$  for the shRNA cells, control value set to %100. **D.** Collagen secretion was measured in a separate series of experiments as in **A** and the results are from  $n = 6$ ,  $*p < 0.05$ . **E.** Western blotting data for the insulin receptor beta subunit following SDS-PAGE were scanned and statistically analyzed ( $n = 4$ ,  $*p < 0.02$ ,  $**p < 0.002$ ).



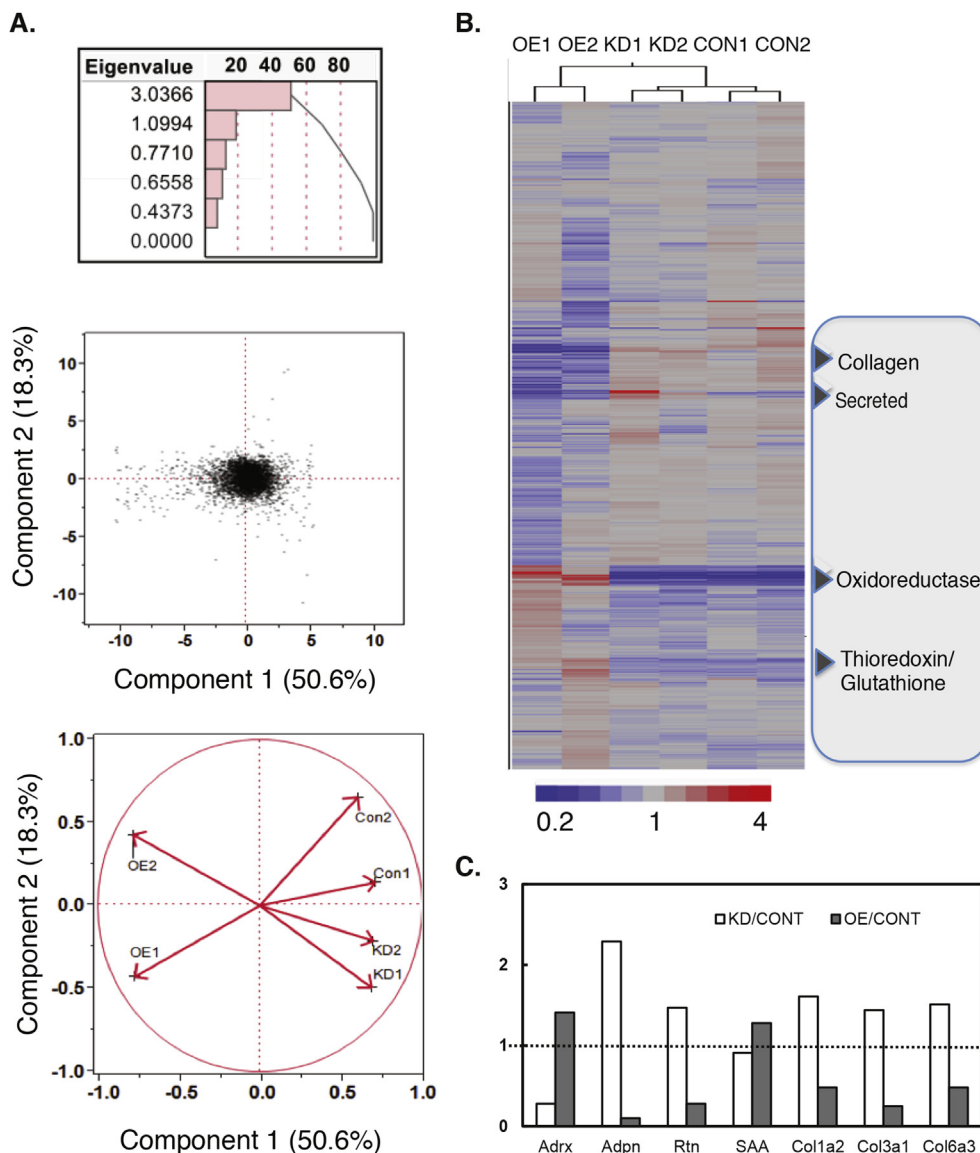
**Figure 3:** Adiporedoxin is upstream of PDI in the adipocyte ER redox cascade. **A.** 3T3-L1 cells over-expressing WT Adrx (CFLC) or with active site cysteines mutated to serines (SFLS) were treated or not with dithiothreitol (DTT), Diamide (Dia.) or not for 5 min, lysed with 20% TCA, and TCA precipitates were reacted with MalPEG-2K to modify free cysteines. Adrx and protein disulfide isomerase a1 (PDIa1) were visualized by immunoblotting following gel electrophoresis of the TCA precipitates and the reduced and oxidized forms indicated. **B.** 3T3-L1 cells expressing scrambled or Adrx-targeting shRNA were treated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 5 min, lysed and treated as in Figure 3A. The bar graph on the right shows the mean  $\pm$  S.D. for scans of 4 independent experiments (\*p < 0.05). **C.** Same as Figure 3B for Adrx over expressing cells and controls (scrambled shRNA).

in the Adrx knockdown cells. On the other hand, serum amyloid A3 protein, which lacks S–S bonds [46], does not change as a result of altered Adrx expression (Figure 4C). The expression of the three specific collagen chains indicated in Figure 4C (see also Supplemental Tables S1 and S2) were affected by alterations in Adrx levels in a manner similar to adiponectin in confirmation of the biochemical analysis of Figure 2D.

### 3.5. *Adrx* null mice show decreased adipokine secretion, have mild ER stress and are hyperinsulinemic

In order to obtain data for the effects of Adrx levels *in vivo*, we introduced into mice ES cells obtained from KOMP for a gene construct that deletes exons 2–6, and we generated *Adrx* knockout mice. The

mRNA expression for WT, heterozygous and *Adrx* null mice is shown in Figure 5A and protein expression data for the WT and knockouts are shown in Figure 5B and quantitatively analyzed in Figure 5C. The mice exhibited increased adiponectin retention and increases in components of the ER protein maturation machinery, BiP and ERO1 $\alpha$  (Figure 5B, C) as well as in a number of mRNAs encoding ER stress proteins and inflammatory markers (Supplemental Figure S2). Mass spectrometric analysis of intact epididymal fat tissue from WT and *Adrx* knockout mice confirmed that adiponectin and other adipokines show higher cellular levels (enhanced retention implying reduced secretion) in the knockout as compared to WT (Supplemental Table S2). The WT and null mice are the same weight (Figure 5D) and body composition (Figure S3B, S3C), and the nulls have decreased levels of circulating



**Figure 4:** Proteomic analysis of tandem mass tagged (TMT)-labeled whole cell (3T3-L1) lysates confirms Western blot data and reveals pleiotropic cellular changes as result of altered Adrx levels. **A.** Principle component analysis (PCA) analyses of the TMT labeling (Table S1) showed good correlation between biological replicates. Eigenvalues and percentages were calculated for each PCA. Graphical representation for each protein by PCA 1 and PCA 2 show the majority of the proteins did not change as a result of altered Adrx levels. Biplots of PCA 1 and 2 shows a vector location of each biological sample where the biological duplicates were found to be most similar to each other. **B.** Heat map showing enrichment of Gene Ontology (GO) categories across Adrx cell lines. Indicated to the right of the heat map are those categories that were significantly enriched with Benjamin-Hochberg corrected  $p < 0.05$ . **C.** Relative amounts of cell associated adipokines (adiponectin, Adpn; resistin, Rtn; serum amyloid a, SAA) and selected collagens (Col1a, 3a, 6a) from Adrx knockdown (KD) and overexpressing (OE) as compared to control (Cont-set to 1.0). The results shown for the TMT experiment compare 2 dishes of each cell line (OE, KD, Cont, see Supplemental Table 1) and the entire experiment was repeated twice with similar results.

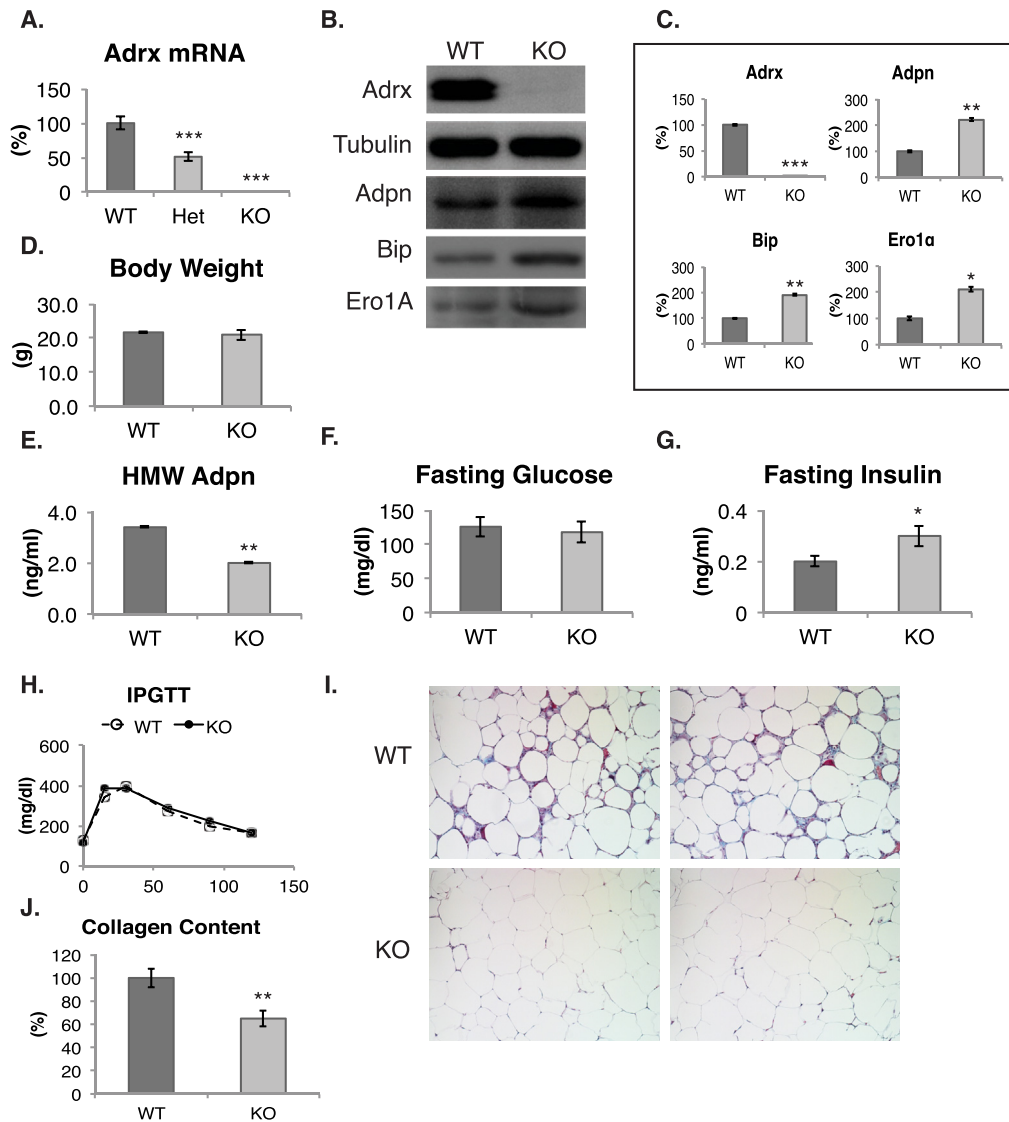
HMW adiponectin (5E) as was expected from the *in vitro* data (Figures 2 and 3). The knockout mice have normal blood glucose (5F) are mildly hyperinsulinemic (5G), but have normal glucose tolerance (5H).

### 3.6. Adrx null mice are protected from fibrosis due to decreased collagen deposition

Remarkably, while the epididymal adipose tissue from WT mice at age months 7 stains strongly for collagen deposition, the epididymal adipose tissue from Adrx null mice is essentially free from fibrosis (Figure 5I), and as determined by mass spectrometry (Supplemental

Table S2), the Adrx null mice had lower levels of 14 collagen isoforms including collagen chains 6a1, a2, a3 & a5, the most abundant species we detected in adipose tissue (see also Figure 4C and [47]). Biochemical analysis of total collagen confirmed the mass spectrometry data (Figure 5J). Thus the *in vivo* collagen expression data are consistent with the adipose tissue staining (Figure 5I) and the results from the *in vitro* mass spectrometry analysis and quantification (Figures 2C and 4C). Interestingly, at age 6–7 months, the Adrx null mice are the same weight and body composition as wild type mice, have identical glucose tolerance but have significantly higher fasting insulin (Supplemental Figure S3).





**Figure 5:** *Adrx* null mice show ER stress, hyperinsulinemia and their adipose tissue is free of fibrosis. **A.** *Adrx* mRNA levels from adipose tissue were examined by RT-qPCR from wild type (WT), heterozygote (Het) and *Adrx* knockout (KO) mice. **B.** Whole adipose tissue lysates from WT and KO mice were prepared in RIPA buffer. Protein (10–50 μg, equal amounts, WT & KO) was subjected to SDS-PAGE and immunoblotted with the indicated antibodies. **C.** Quantification of Adrx, Adpn, Bip and Ero1α from **6B**, body weight (**D**), fasting serum insulin (**E**) and glucose (**F**), HMW adpn (**G**), and glucose tolerance test (**H**) were assessed in WT and *Adrx* KO mice. **I.** Collagen Masson's Trichrome Stain and **J.** collagen quantification by using Sirius Red Total Collagen Detection Kit of adipose tissue from WT and *Adrx* KO mice. 8-week-old strain-matched males were used in (a–h), n = 3, and 7 months old mice were used in (i–j), n = 6. The statistical values are displayed as means and S.E. (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).

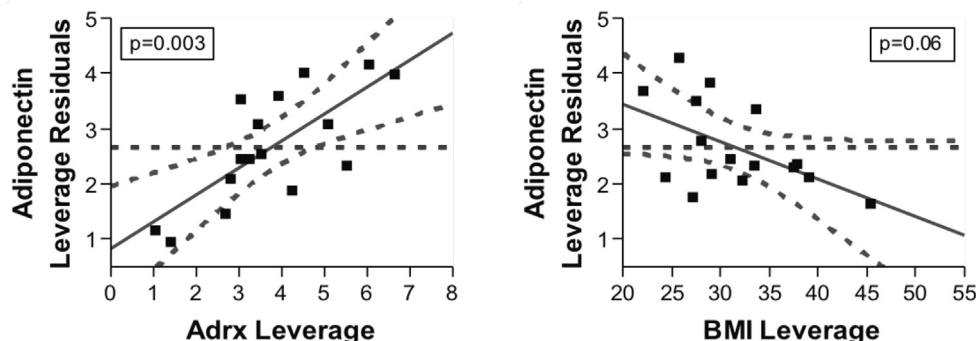
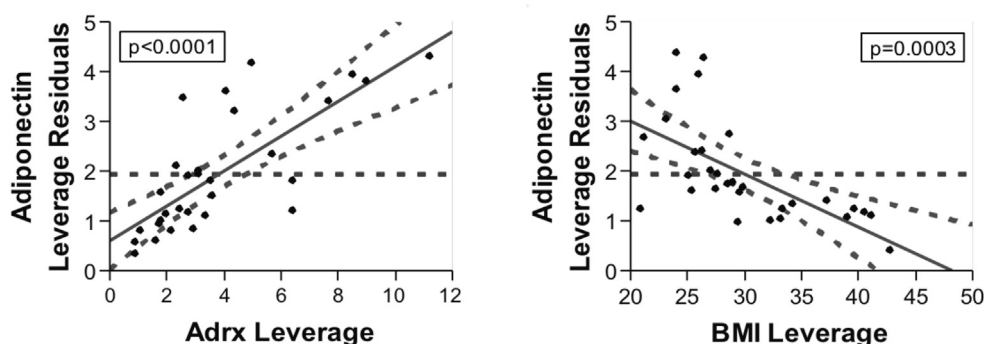
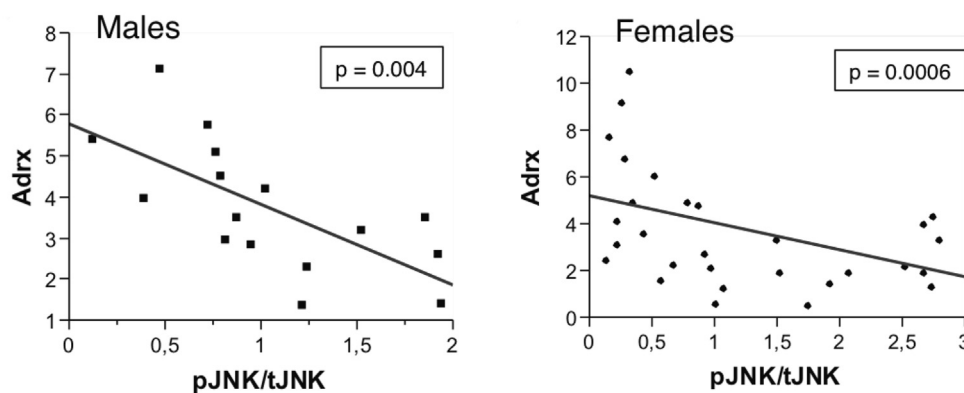
### 3.7. *Adrx* is expressed in human adipocytes and down regulated in proportion to the level of inflammation

*Adrx* mRNA in human adipose tissue was tightly correlated with adipose tissue adiponectin mRNA in both sexes (Figure 6A, males, 6B, females) after statistical adjustment for BMI, which had a negative effect on adiponectin mRNA (Figure 6A, B, right panels). A similar correlation was noted for *Adrx* protein and tissue adiponectin, independent of BMI  $R^2 = 0.53$ ,  $p < 0.01$  in males;  $R^2 = 0.49$ ,  $p < 0.0001$  females, data not shown). In addition, tissue *Adrx* protein levels were inversely related to the ratio of pJNK to total JNK protein for fat cells of both sexes (Figure 6C), pJNK being a marker of inflammation in human adipose tissue [48], and this association was independent of BMI in multiple regression models (data not shown). Lastly, male wild type mice fed a high fat diet showed elevated adipose inflammation [49] and also showed reduced *Adrx* levels and reduced circulating

adiponectin, whereas females do not show any changes in these parameters (Supplemental Figure S4).

## 4. DISCUSSION

We describe herein the actions of adiporedoxin (*Adrx*), a novel protein highly enriched in adipocytes and localized to the endoplasmic reticulum, whose altered expression *in vitro* in mouse and human cells, and *in vivo* in mice, results in significant changes in adipocyte protein secretion. In particular, its knockdown in mouse and human fat cells resulted in a significant attenuation of adiponectin secretion (Figures 2, 4 and S1), a protein whose expression has been linked with insulin sensitivity [50] and the secretion of collagens (Figures 2, 4 and 5 and Supp. Table 2), proteins which can cause adipocyte fibrosis and insulin resistance [19,51]. Both types of proteins require disulfide bonds for

**A. Males**

**B. Females**

**C.**


**Figure 6:** Expression of adiponectin and adiporedoxin mRNA positively and strongly correlate independent of BMI and Adrx protein negatively correlates with phospho-Jun kinase (pJNK) levels. Figure 6 shows mRNA levels of Adrx and adiponectin in men (**A**) and women (**B**). Associations were analyzed in a least squares model that including the interaction term  $Adrx \times BMI$  which was significant in the females ( $p = 0.01$ ) but not in the males ( $p = 0.12$ ). The model predicted 60% of the adiponectin variance in the females ( $R^2 = 0.60$ ,  $n = 30$ ,  $p < 0.001$ ), and 62% in males ( $R^2 = 0.62$ ,  $n = 16$ ,  $p < 0.01$ ). **C.** Quantitative analysis of Western blots of adipocyte lysates for the ratio of p-JNK to total JNK, plotted *versus* Adrx for both sexes, shows a negative correlation in both males ( $r = -0.68$ ,  $n = 16$ ) and female subjects ( $r = -0.49$ ,  $n = 29$ , one outlier excluded).

assembly and secretion. Moreover, we showed by proteomic analysis that Adrx over and under expression enhanced and attenuated, respectively, the secretion of several S–S bonded adipokines including resistin (Figure 4) and adipisin (Western blot not shown) as well as affected insulin receptor amounts (Figure 2), the latter presumably due to its large number of disulfide linkages [39]. As most secreted proteins have disulfide bonds, appropriate controls are scarce for the measuring the effects of Adrx on secreted proteins lacking disulfides. However, serum amyloid A protein is such a protein [46], and its secretion is in fact unchanged in by manipulation of Adrx expression

(Figure 4). Importantly, we confirmed much of the protein secretion data in studies of *Adrx* null mice (Figure 5), which circulate lower levels of HMW adiponectin and are remarkably free of fibrosis due to impaired collagen secretion. Lastly, Adrx protein levels in human fat tissue correlate well with adiponectin protein levels, and inversely with cellular inflammatory markers regardless of BMI. Thus higher levels of Adrx contribute to the maintenance of healthy adipose tissue despite obesity (Figure 6).

The data we obtained are consistent with Adrx acting at an early step in the secretory pathway of adipocytes. Thus reduced Adrx expression in

the context of ER oxidative stress (hydrogen peroxide, Figure 3) results in impaired redox regulation of PDIa1, the most abundant adipocyte chaperone found in our proteomic analysis of mouse adipose tissue (Supp. Table 2), which is directly involved in disulfide bond formation [21]. Adrx appears to be in the peroxiredoxin (Prx) family of redox-regulated proteins [52] that have diverse biological functions in a variety of cell types, reduction of peroxides being their eponymous activity [53]. The only other endoplasmic reticulum localized peroxiredoxin, Prdx4, functions in the formation of disulfide bonds indirectly, probably by regulating the amount of reactive oxygen species, principally hydrogen peroxide [54,55]. As noted, disulfide bond formation is directly mediated by protein disulfide isomerases (PDIs) whose active sites have two or three -CGHC- sequences along with a chaperone fold consisting of about 130 amino acids with four beta sheets and 3 alpha helices [21]. The PDIs bind to substrates, form and if necessary, reform and correct their disulfide bonds, although many details of the process remain unclear [34]. On the other hand, adiporedoxin has a unique thioredoxin active site sequence, -CFLC-, not found in other PDI/Trx proteins, nor in the other peroxiredoxin family members, including Prdx4, as peroxiredoxins have cysteines widely separated in their primary protein sequences and function as multimers [53]. Moreover, unlike most members of the PDI family and Prdx4, which are soluble in the lumen of the organelle, Adrx is a transmembrane protein (Figure 1) similar to the transmembrane thioredoxin-like protein 4 (TMX4) only with respect to its ER membrane location [40,56].

Based on the data of Figure 3, we hypothesize that Adrx has a function unique to adipocytes that likely involves its ability to deal with metabolically related ER oxidative stress and to mediate protein secretion under these conditions. Recent related work on the role of various ER proteins involved in oxidative protein folding and disulfide bond formation has revealed redundancies and complementary roles for multiple PDI/Trx-related proteins [34,55], fourteen of which are expressed in the adipocyte ER (Supplemental Tables S1, S2, see also [47]). The relatively modest metabolic phenotype of the *Adrx* null mice, similar in that respect that to the *ERO1 $\alpha$ /ERO1 $\beta$*  knockout [57], likely reflects this complexity and redundancy in ER oxidoreductase function. On the other hand, the biochemical phenotype of the *Adrx* null mouse with regard to collagen secretion is particularly robust. Thus, considerable additional work will be required to define the biochemical role of Adrx more precisely and its hierarchical role in adipocyte ER redox regulation, and this is in progress. Adrx has previously been reported in one publication naming it peroxiredoxin 2-like protein expressed in bone marrow monocytes (PAMM, also known as Fam213a) [52] that affects cellular redox status, a result consistent with the present data. Its expression in fat tissue was not examined in the cited study, but the authors showed protein to be expressed in human liver, brain and other tissues, at what appears to be one or more orders of magnitude lower than in adipocytes, as compared to our data on Adrx levels in these mouse tissues (Figure 1). These authors showed a minor extent of PAMM mRNA upregulation and protein in macrophage colony-stimulating factor activated bone marrow monocytes *in vivo*, but subsequent verification of its redox function was performed in transfected cells *in vitro*. Thus a physiological function *in vivo* for PAMM in monocytes was not definitively established.

A second aspect of Adrx function in adipocytes concerns the biochemical nature of the most abundant secreted molecules of these cells, adiponectin and collagen. These proteins are particularly interesting, not only for reasons of their abundance and important physiological role(s), but also for the fundamental biochemistry and cell biology of their complicated quaternary structures, post translational

modifications including intermolecular disulfide bond formation [16,58]. The adiponectin trimer consists of an intermolecular disulfide link between 2 monomers and a free SH group on the 3rd subunit, which is then disulfide bonded to another trimer to form the hexamer [12,59,60]. Likewise, intrachain disulfide bonds between the N- and C-terminal propeptide sequences are necessary to form the signature triple helix structure of collagens [58] before their movement out of the ER. The striking biochemical phenotype of the *Adrx* knockout mouse emphasizes Adrx's roles as a particular modulator of collagen secretion and that of related proteins. Regarding adiponectin, previous studies have documented a direct interaction of an adiponectin cysteine residue with one in ERp44, which bond can be released by reducing agents or by increasing *ERO1 $\alpha$*  levels [24]. This mechanism, enhanced retention, is analogous to the assembly of IgK [61,62]. However, adipisin has only intermolecular disulfides [63] and the insulin receptor has both types of bonds [39]. This would support the above stated notion that Adrx plays a broad role early in disulfide bond formation, e.g. ER redox status, such that a compromise in its activity reduces the overall levels of most or all disulfide bonded integral membrane and secreted proteins. DsbA-L was shown to directly interact with adiponectin in yeast-2 hybrid studies and to promote its multimerization [26]. DsbA-L was also shown to alleviate the effects of adipocyte ER stress on adiponectin levels [64], but it does not appear to be a luminal ER resident (Figure 2), presumably a requisite for a direct role in oligomer assembly, and its knockout in mice is without effect on adiponectin multimerization [65]. Thus a further examination, *in vitro* and *in vivo*, of the possible players in adipocyte protein secretion is needed to resolve the specific roles of these proteins in the formation of adipokines and integral membrane proteins and to further elucidate their mechanism(s) of action.

Given its pleiotropic action on adipokines and other secreted proteins related to insulin sensitivity, the relationship of Adrx levels to pathophysiological states in mice and humans is likely to be complex. Indeed, we see no dramatic effects of Adrx deficiency on systemic glucose utilization in null mice on a chow diet (fasting glucose and glucose tolerance, Figure S3). However, lower circulating HMW adiponectin levels and mild hyperinsulinemia would suggest some degree of insulin resistance. Moreover, fibrosis, while present in WT animals, is virtually absent in the epididymal fat pad of older Adrx deficient mice, and this should enhance the metabolic flexibility and the insulin sensitivity of adipose tissue and the entire mouse and, therefore, could compensate for the effects of hypo adiponectinemia and hyperinsulinemia at least with regard to glucose tolerance. In human adipose tissue we also found a close correlation between Adrx and adiponectin levels in both men and women, independent of BMI. Furthermore, higher expression of activated (phosphorylated) JNK, a marker of ER stress, inflammation and insulin resistance [48] was inversely correlated with Adrx protein (Figure 6C). In WT male mice following 12 weeks of a high fat diet, which results in insulin resistance, reduced adipocyte Adrx protein correlates with lower circulating adiponectin (Supplemental Figure S4). Taken together, the data from mice and humans point to a positive effect of Adrx protein levels in ameliorating insulin resistance, although the relative contribution(s) in this regard of adipocyte secretory proteins downstream of this protein remain to be fully elucidated. Thus, we postulate that Adrx may be down regulated in response to the metabolic and inflammatory status of adipose tissue and this mechanism contributes to lower adiponectin levels in obese humans, which is likely to be one of several factors influencing the metabolic status of the individual. In summary, adiporedoxin is clearly an important contributor to fat cell biology in mice and humans and one that participates in and regulates novel aspects of the secretory

pathway in these cells of relevance to basic cellular functions, organismal metabolism and pathology.

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## CONFLICT OF INTEREST

The authors declare no competing financial interests.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molmet.2015.09.002>

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