The Rho GTPase RND3 regulates adipocyte lipolysis

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Background: Adipose tissue plays a crucial role in diet- and obesity-related insulin resistance, with implications for several metabolic diseases. Identification of novel target genes and mechanisms that regulate adipocyte function could lead to improved treatment strategies. RND3 (RhoE/Rho8), a Rho-related GTP-binding protein that inhibits Rho kinase (ROCK) signaling, has been linked to diverse diseases such as apoptotic cardiomyopathy, heart failure, cancer and type 2 diabetes, in part by regulating cytoskeleton dynamics and insulin-mediated glucose uptake.

Results: We here investigated the expression of RND3 in adipose tissue in human obesity, and discovered a role for RND3 in regulating adipocyte metabolism. In cross-sectional and prospective studies, we observed 5-fold increased adipocyte levels of RND3 mRNA in obesity, reduced levels after surgery-induced weight loss, and positive correlations of RND3 mRNA with adipocyte size and surrogate measures of insulin resistance (HOMA2-IR and circulating triglyceride/high-density lipoprotein cholesterol (TAG/HDL-C) ratio). By screening for RND3-dependent gene expression following siRNA-mediated RND3 knockdown in differentiating human adipocytes, we found downregulation of inflammatory genes and upregulation of genes related to adipocyte lipolysis and insulin signaling. Treatment of adipocytes with tumor necrosis factor alpha (TNFα), lipopolysaccharide (LPS), hypoxia or cAMP analogs increased RND3 mRNA levels 1.5–2-fold. Functional assays in primary human adipocytes confirmed that RND3 knockdown reduces cAMP- and isoproterenol-induced lipolysis, which were mimicked by treating cells with ROCK inhibitor. This effect could partly be explained by reduced protein expression of adipocyte triglyceride lipase (ATGL) and phosphorylated hormone-sensitive lipase (HSL).

Conclusion: We here uncovered a novel differential expression of adipose RND3 in obesity and insulin resistance, which may at least partly depend on a causal effect of RND3 on adipocyte lipolysis.

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1. Introduction

Obesity-related risk of morbidity and mortality [1] involves systemic insulin resistance linked to inflammation in adipose tissue [2,3], which may largely distinguish people with metabolically “unhealthy” and “healthy” obesity [4]. Increased adipose tissue inflammation further associates with elevated circulating free fatty acids (FFA), visceral and ectopic lipid accumulation and lipotoxicity, likely in large part due to increased adipocyte lipolysis [5–8]. Identification of novel genes and pathways that mediate altered adipose tissue function in obesity is therefore of great interest, and may enable development of new therapeutic strategies.

Lipolysis in adipocytes is critical for lipid handling and systemic metabolic homeostasis [9]. Adipocyte lipolysis increases during fasting, exercise and other factors to release FFA for use by other tissues and organs, in part induced by beta-adrenergic stimuli. These stimuli induce the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, and consequently activate lipolytic enzymes, most notably hormone-sensitive lipase (HSL) by phosphorylating activity-
controlling serine residues (i.e., ser650, corresponding to ser651 in mouse and ser660 in rat) [10–12]. Additional pathways also stimulate lipolysis via HSL and/or other enzymes [13], such as activation of adipose triglyceride lipase (ATGL) by 5′ adenosine monophosphate-activated protein kinase (AMPK) [14]. Feeding and increased insulin levels inhibit these processes in a normal physiologic regulation [9]. However, while short-term overfeeding (for a few days) does not provoke dysregulated lipolysis in adipocytes [15], conditions of chronic nutrient stress over weeks and months may increase lipolysis in conjunction with developing insulin resistance in adipocytes. Hence, a partial reduction in lipolysis may enhance glucose metabolism [6]. Genetic evidence and different experimental models further support that genotype-dependent increases in lipolysis may cause insulin resistance and metabolic diseases by promoting visceral and ectopic fat accumulation [7,16], although de novo lipogenesis and impaired lipid handling in adipocytes may also contribute [17].

RND3 (Rho-related GTP-binding protein RhoE/Rho8) is a member of the Rnd family, which represents a sub-group of the Rho family of small GTP-binding proteins (G-proteins). The Rnd family also includes Rnd1 (Rho6) and Rnd2 (Rho7) [18]. Most small G-proteins are molecular switches, which cycle between a non-active, resting GDP-bound form and an active.

GTP-bound form [18]. However, unlike RND1 and RND2, RND3 with its unique structure [19] has no intrinsic GTPase activity and is insensitive to the Rho-specific GTPase-activating proteins (GAPs) [20]. Through its basic function in regulating the formation of actin stress fibers and cytoskeleton dynamics, RND3 has been implicated as a driving factor in the pathophysiology of diseases such as apoptotic cardiomyopathy, heart failure, and cancer [21]. RND3 acts in part by inhibiting the activity of RhoA and its downstream effector Rho-associated coiled-coil containing kinase 1 (ROCK1), signals that drive stress fiber formation [22]. RND3 was previously shown to impair insulin-mediated glucose uptake in skeletal muscle of patients with obesity and T2D through direct binding and inhibition of ROCK1 [23]. Furthermore, in gastric cancer cells, hypoxia-inducible factor-1α (HIF-1α), a transcription factor implicated in adipose tissue insulin resistance and inflammation [24,25], was found to bind a hypoxia-responsive element (HRE) in the RND3 promoter to induce RND3 expression [26]. These data point to a possible role for RND3 in regulating adipose tissue function. We here hypothesized that RND3 regulates adipose tissue metabolism and function in humans, with a possible role in obesity and insulin resistance.

2. Methods

2.1. Ethics and Subjects

The study was approved by the Regional Ethics Committee (REK Vest, Norway, approval numbers 2010/512 and 2010/3405, and the Regional Committee of Ethics in Stockholm, Sweden). Each subject gave written informed consent. Blood and adipose tissue biopsies were collected from three cohorts (Cohorts 1–3, Table 1).

2.2. Adipose Tissue Sampling

Human adipose tissue was analyzed for three different cohorts. Subcutaneous (SC) and omental (OM) adipose tissue biopsies were obtained by surgical excision from patients with severe obesity undergoing bariatric surgery in Western Norway (Førde Hospital and Voss Hospital), as previously described [27,28] (Cohorts 1–2, Table 1). Subcutaneous biopsies were also obtained from a subset of patients one year after bariatric surgery and from non-obese healthy people (Cohort 1) [27]. In Cohort 2 [28], adipocytes and the stromal vascular fraction (SVF) were isolated from SC adipose tissue. In Cohort 3 [29], subcutaneous adipose tissue samples were collected by needle biopsy from the periumbilical region of non-obese (n = 26) and obese (n = 30) women. Clinical details of Cohort 3 as well as sample preparation and gene microarrays are described in the original publication [29]. The samples were collected between 2003 and 2010.

2.3. Adipose Tissue Homogenization and Fractionation

Frozen whole tissue (200–300 mg) was homogenized in a 2 mL safe-lock eppendorf tube with 1 mL Qiazol lysing buffer (Qiagen) and a 5 mm metal bead (Millipore), using a TissueLyser (Qiagen) with three repeated shakings at 25 Hz for 2 min each. To isolate adipocytes and SVF, 700–800 mg of adipose tissue was treated with collagenase and thermolysin (Liberase Blendzyme 3, Roche), and completed within 1 h ± 5 min, as previously described [30]. The tissue/cells were frozen immediately and stored at −80°C for later gene expression analysis.

2.4. Adipocyte Cultures

Primary human adipose stromal cells (hASCs) were isolated from subcutaneous liposuction aspirate, cultured and differentiated, as described previously [30]. 3T3-L1 cells were cultured in 24-wells plates in DMEM 4.5 g/L glucose containing 1% penicillin and streptomycin (PEST), at 37°C, as described previously [31]. For the hypoxia experiment, the adipocytes were placed in an incubator with an atmosphere of 2% oxygen, 5% CO2 and 93% N2, to be compared with the standard atmosphere (5% CO2, 21% oxygen and 74% N2). Human preadipocytes from the human SGBS adipocyte cell line [32] were cultured and propagated in proliferation medium consisting of DMEM/Ham’s F-12 (1:1) supplemented with 10% fetal calf serum (FCS), 33 μM biotin, 20 μM DL-pantothenate, 100 U/mL penicillin and 100 μg/mL streptomycin. Differentiation was induced with DMEM/F12 medium containing 10 μg/mL transferrin, 66 nM insulin, 1 nM triiodothyronine (T3) and 100 nM cortisol. During the first 4 days of differentiation, the medium was also

| Cohort | M/F | Age (years) | BMI (kg/m²) | HOMA2-IR | TAG/HDL | Figure |
|--------|-----|------------|-------------|-----------|----------|--------|
| Lean   | 7/6 | 44.0 ± 17.5| 23.0 ± 2.5  | 1.45 ± 0.63 | 0.83 ± 0.45 | 1B     |
| Obese  | 4/12| 39.4 ± 10.9| 53.3 ± 4.3  | 3.0 ± 1.65 | 1.48 ± 0.74 | 1C–D, 2A|
| Post-surgery | 4/12 | 40.3 ± 10.9 | 33.1 ± 5.0 | 1.63 ± 0.64 | 1.50 ± 1.18 |       |
| Lean   | 5/7 | 43.5 ± 11.6| 22.8 ± 2.1  | 1.11 ± 0.40 | 0.96 ± 0.67 | 1C–D, 2A|
| Obese  | 4/8 | 43.2 ± 9.98| 43.7 ± 5.2  | 2.38 ± 1.00 | 2.36 ± 1.09 | 1C–D, 2A|
| Post-surgery | 1/5  | 43.8 ± 9.13 | 32.9 ± 5.7 | 1.32 ± 0.38 | 0.95 ± 0.36 | 1C–D, 2A|
| Non-obese/obese | 0/56 | 42.9 ± 12.0 | 33.1 ± 9.9  | 1.38 ± 1.15 | N/A    | 2B     |

M/F, male/female; BMI, body-mass index (kg/m²); OM, omental adipose tissue; Pre, before bariatric surgery; Post-surgery, one year after bariatric surgery/after profound fat loss; HOMA2-IR, homeostatic model assessment 2 of insulin resistance; TAG, triacylglycerol; HDL, high-density lipoprotein cholesterol.

* n = 6 due to missing data.
supplemented with 500 μM 3-isobuty1-1-methylxanthine, 25 nM dexamethasone and 2 μM rosiglitazone. For some experiments, cells were treated with the cAMP analogs 8CPT-cAMP or dibutyryl cAMP (dbcAMP) (activators of PKA as well as Exchange protein activated by cAMP (Epac) signaling), 6 MB-cAMP (activator of PKA signaling), isoproterenol (β3-adrenergic receptor agonist) or ROCK inhibitor (Y27632, selective inhibitor of p160ROCK).

2.5. siRNA Transfection

After differentiation for up to 11 days, hASCs were transfected for 48–72 h with 25 nM ON-TARGETplus human siRNA SMARTpool (Dharmacon, Thermo Fisher Scientific) using HiPerFect (Qiagen) (non-targeting siRNA control and siRNA against RND3 (L-007794-00-0005) or HIF1A (J-004018-07). Cells were collected in buffer RLT (Qiagen) and stored at −80°C until RNA extraction.

For the lipolysis assay (see below), hASCs were transfected at day 8 of differentiation with siRNAs targeting RND3 or a scramble non-targeting siRNA pool using Neon electroporator (Invitrogen) according to the manufacturer’s protocol. Briefly, for one transfection reaction 1 million of hASCs suspended in 90 μL of R buffer were mixed with 200 pmol/μL of siRNA and transfection was performed using a 100 μL electroporation tip. Electroporation conditions were 1400 Volts, 20 ms width, and 2 pulses. Following electroporation the cells were plated in 48-well plates at a density of 55,000 cells/well in 250 μL medium, with a final siRNA concentration of 40 nM. The next day medium was replaced to the fresh medium with reduced insulin (0.01 nM), and ROCK inhibitor was added at a final concentration of 10 μM, followed by incubation of the cells for 72 h. Each sample was prepared in quadruplicates and the experiment was repeated three times. 

2.6. RNA Extraction

The RNeasy Lipid Tissue Midi Kit (whole tissue) or Mini Kit (Qiagen) was used to extract total RNA. NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer were used to measure amount (ng/μL) and quality (RNA integrity number) of RNA.

2.7. qPCR Analysis

cDNA was prepared from 1 μg total RNA by the Transcriptor First Strand cDNA Synthesis Kit (Roche), and diluted 1:10 with PCR-grade water. For the cell fractions and cell culture, the SuperScript® VlO™ cDNA Synthesis Kit (Invitrogen) was used, with an input of 100 ng (cell fraction) and 500 ng (cell culture) total RNA per sample, followed by 1:20 dilution with PCR-grade water. The LightCycler480 Probes Master kit and the LightCycler480 rapid thermal cycler system (Roche Applied Science) were used to perform qPCR. Target and reference genes were amplified by specific primers and Universal ProbeLibrary (UPL) probes (Roche) (Table 2). Amplification efficiency based on standard curves was used to calculate mRNA concentrations. TBP and IP08 [33] were chosen as reference genes based on their stable expression in biopsies and primary culture, respectively, and because they showed similar expression levels as the target genes.

2.8. Microarray Analysis

The Illumina microarray analyses were performed as described previously [27]. Briefly, 300 ng of total RNA from each sample was reversely transcribed, amplified and Biotin-16-UTP–labeled. NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer were used to measure amount (15–52 mg) and quality of the labeled cRNA. 750 ng of biotin-labeled cRNA was hybridized to the HumanRef-8.v3 (whole tissue) or HumanHT-12.v3 Illumina Sentrix BeadChip according to manufacturer’s instructions. The Affymetrix microarray data from Cohort 3 were analyzed as described in detail previously [29].

2.9. RNA Sequencing

cDNA libraries were generated via the TruSeq Stranded mRNA Library Prep kit. RNA-seq was then performed at the Illumina Hiseq 4000 platform at the Genomics Core Facility, Bergen. Aligned reads were put into featureCounts (Version 1.5.2) with options “featureCounts -T 64 -p -t exon -g gene_id”, resulting in a matrix of raw counts. Data were normalized and differential expression analysis was performed with DESeq2 (Version 1.22.2). For pathway enrichment analysis we used the KEGG database. Significant ontologies were visualized using ggplot2 package (Version 3.1.0). PCA plots were generated using the R package ‘DESeq2’ (Version 1.22.2) and the Volcano plot of the differential expression analysis by the R package ‘Enhanced Volcano’ (Version: 1.0.1).

2.10. Lipolysis in In Vitro Differentiated Adipocytes

Glycerol in conditioned media was measured using Free Glycerol Reagent (Sigma Aldrich) and Amplex UltraRed (Invitrogen) according to the manufacturer’s instructions. Amplex Ultra Red was diluted 100-fold in Free Glycerol Reagent, mixed with 20 μL of conditioned medium in a 96-well plate, incubated at room temperature for 15 min, and fluorescence was measured (excitation/emission 530/590) using an Infinite M200 plate reader (Tecan Group, Männedorf, Switzerland). The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) and protein concentration was assessed using BCA Protein Assay Kit (Thermo Fisher Scientific). Glycerol values were normalized to the proteins in each well.

2.11. SDS-PAGE and Immunoblotting

Fully differentiated SGBS cells (day 15) were transfected with 25 nM non-targeting or RND3-targeting siRNA for 72 h, and treated with 10 μM

| Gene name | Organism | Primer sequence | UPL probe |
|-----------|----------|-----------------|-----------|
| RND3      | Human    | F: 5′-gcctgtgctcagttcctc-3′  | 38        |
|           |          | R: 5′-gcctgtgctcagttcctc-3′  |           |
| HIF1A     | Human    | F: 5′-tcacaactttcaagctgagta-3′ | 74        |
|           |          | R: 5′-tcacaactttcaagctgagta-3′ |           |
| IP08      | Human    | F: 5′-tggctctctctctctctctct-3′ | 11        |
|           |          | R: 5′-tggctctctctctctctctct-3′ |           |
| TBP       | Human    | F: 5′-tcataactaatggagagagagg-3′ | 38        |
| Rnd3      | Mouse    | F: 5′-tccataagttaacagcagagg-3′ | 38        |
| RpLp0     | Mouse    | F: 5′-tccataagttaacagcagagg-3′ | 9         |

Table 2

Primer sequences used for qPCR.

The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) and protein concentration was assessed using BCA Protein Assay Kit (Thermo Fisher Scientific). Glycerol values were normalized to the proteins in each well.
ROCK inhibitor for the last 24 h of the transfection. Post transfection, protein samples were prepared in RIPA buffer with phosphatase (phosSTOP; Roche) and protease inhibitors (cOmplete; Roche). After centrifugation (20,000 g, 4°C, 15 min), the fatty supernatant was removed, and protein concentrations were assessed by Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific). Denatured protein samples were analyzed by SDS-PAGE and immunoblotting using mouse anti-RND3 (1:500, Cell Signaling#3664), rabbit anti-HSL (1:750, Cell Signaling#4107), rabbit anti phosphorylated (p) HSLser660 (human ser650) (1:750, Cell Signaling#4126) or rabbit anti-ATGL (1:750, Cell Signaling#2138) antibodies.

2.12. Statistics

P-values were calculated by ANOVA and Tukey's posthoc test or t-test, as indicated. Correlations were calculated by Pearson's correlation. These statistics were performed in Excel for Windows 10. For the Illumina microarray analyses we used Significance Analysis of Microarrays (SAM) to identify differentially expressed genes, as described previously [27]. Details on the statistical analysis of the Affymetrix microarrays in Cohort 3 are described in [29]. For the RNA sequencing, differentially expressed genes identified by DESeq2 (Version 1.22.2) (false discovery rate (FDR) cutoff <0.1) were used as an input and Fischer's exact test was used to identify statistically over/under represented pathways (FDR cutoff <0.05).

3. Results

3.1. Up-regulation of RND3 in Obesity and Metabolic Syndrome

To examine the expression of RND3 in adipose tissue, we first analyzed publicly available transcriptome data for two strains of mice with genetically induced obesity due to leptin deficiency (ob/ob), i.e., obesity-prone C57BL/6 (B6) and the insulin resistant-prone Black and Tan BRachyury (BTBR) [34]. In comparison to other metabolic tissues, adipose tissue showed the highest Rnd3 mRNA expression and increased levels in the obese and insulin resistant mice compared to controls after 4 weeks of feeding, and also after 10 weeks in the BTBR model (Fig. 1A). The increased expression already at 4 weeks of overfeeding in the BTBR model is consistent with a primary effect of Rnd3 on insulin resistance and consequent diabetes, since these mice are insulin resistant at all ages independent of obesity but develop severe diabetes first after 10 weeks [34]. Although showing the lowest expression, pancreatic islets expressed significantly less Rnd3 in both

Fig. 1. Elevated adipose RND3 mRNA expression in obesity. A. Lean and obese (ob/ob) B6 and BTBR mice were fed for 4 or 10 weeks (ad libitum). Adipose tissue (epididymal), liver, hypothalamus, soleus, gastrocnemius and pancreatic islets were collected (n = 5). Data are presented as mean ± SD. B. mRNA signal intensities were extracted from a microarray adipose transcriptome dataset (Illumina BeadChips, Cohort 1). Data are presented as mean ± SD (lean n = 13, subcutaneous adipose tissue obese n = 16, subcutaneous post-surgery n = 16, omental obese n = 12). Adipocytes and stromal vascular fraction (SVF) were isolated by digesting fresh human adipose tissue with collagenase. mRNA signal intensities were extracted from a microarray transcriptome dataset (Illumina BeadChips). Data are presented as mean ± SD (n = 12 per group). D. For a subset of the patients, adipocytes were isolated from subcutaneous adipose tissue also after profound fat loss (one year after bariatric surgery). RND3 mRNA was measured by qPCR and related to TBP mRNA (reference gene). Data are presented as mean ± SD (n = 6). P-values were calculated by one-way ANOVA and Tukey's posthoc test, except panel D (paired t-test). B6, C57BL/6 inbred mouse strain; BTBR, Black and Tan BRachyury mouse strain; Ob, obese; Ob/ob, obese leptin-deficient; OM, omental adipose tissue; Pre, before bariatric surgery; Post, one year after bariatric surgery; SC, subcutaneous adipose tissue; SVF, stromal vascular fraction. * p < .05; ** p < .01; *** p < .001.
strains at 4 weeks. On the other hand, muscle (gastrocnemius) showed opposite effects in the two strains after 10 weeks (Fig. 1A).

We further compared RND3 mRNA expression in whole adipose tissue biopsies from 13 lean and 16 extremely obese insulin resistant or diabetic Caucasians (Cohort 1, Table 1) [27]. Relative to lean samples, obese samples showed a 6-fold increase in RND3 mRNA in abdominal subcutaneous fat, and a return to normal levels after profound fat loss due to bariatric surgery (average BMI of 33 one year after surgery) (Fig. 1B). RND3 also showed a notable expression in omental adipose tissue from the same extremely obese patients, around 35% lower than in subcutaneous fat (Fig. 1B). In comparison, adipose expression levels of RND1 and RND2 were around 10-fold lower than those of RND3, and showed no alterations in obesity (Fig. S1A).

Because adipose tissue harbors a heterogeneous population of cells, including adipocytes, progenitor cells and immune cells, we measured RND3 mRNA expression separately in adipocytes and stromal vascular fraction (SVF) isolated directly from subcutaneous (lean and obese) and omental (obese) adipose tissue (n = 12 in the lean and obese groups, Cohort 2, Table 1). RND3 mRNA was expressed ~2-fold higher in isolated adipocytes from obese compared to lean samples, while we observed no such obesity-dependent expression in SVF (Fig. 1C). Consistent with the whole tissue data, omental adipocytes and SVF from the obese patients showed 40–60% lower RND3 expression than subcutaneous adipocytes collected from the same patients (Fig. 1C). We also measured changes in RND3 mRNA in isolated subcutaneous adipocytes collected from a subset of these patients before and after profound fat loss, and found that adipocyte RND3 mRNA was halved (Fig. 1D), completely reversing the 2-fold higher level seen in obesity relative to lean patients (Fig. 1C).

We further examined whether RND3 expression in isolated adipocytes might relate to whole-body insulin sensitivity and circulating lipids. We first correlated subcutaneous RND3 mRNA across the lean and obese patients with HOMA2-IR and TAG/HDL ratio. While RND3 mRNA was measured by microarrays (Illumina BeadChips) in subcutaneous and omental adipose tissue (Fig. 2A), we observed a similar pattern in isolated omental adipocytes (Fig. 2A). Another cohort of 56 Caucasians (Cohort 3, Table 1) confirmed significant positive correlations for RND3 mRNA in subcutaneous whole tissue with BMI and HOMA2-IR (r = 0.33, p = 0.012) (Fig. 2B), as well as with waist-to-hip ratio (WHR) (r = 0.29, p = 0.034) (not shown). Moreover, consistent with the relationship of adipose RND3 with phenotypes related to insulin resistance, RND3 correlated inversely with plasma glucose disappearance rate (KITT based on an insulin tolerance test) (r = −0.39, p = 0.027) (not shown).

To assess whether adipose RND3 might contribute to these metabolic phenotypes via adipocytes, we correlated RND3 mRNA with subcutaneous adipocyte morphology (number and size). While RND3 showed no significant correlation with adipocyte number, we observed a strong positive correlation with subcutaneous adipocyte volume (r = 0.47, p = 0.0002) (Fig. 2B), and with hypertrophic fat cell morphology defined by the morphology value, i.e. the relative level of adipocyte hypertrophy or hyperplasia at any given body fat mass [35] (r = 0.3, p = 0.02). By multiple regression analysis we found that the positive association with adipocyte size remained significant after correcting for BMI (β = 0.19, p = 0.047), despite a strong correlation between RND3 and BMI (β = 0.67, p < 0.0001). None of the other variables showed a significant correlation with RND3 mRNA after BMI adjustment.

3.2. Adipose RND3 is Co-expressed with Pro-inflammatory Genes

The strong relationship between RND3 expression and obesity-related metabolic features prompted us to explore functional roles of RND3 in adipose tissue. To this end, we first performed co-expression analyses to predict cellular processes associated with altered RND3 expression in vivo, for subcutaneous and omental whole adipose tissue (Cohort 1) and isolated adipocytes (Cohort 2) from patients with morbid obesity. Genes positively co-expressed with RND3 consistently showed a significant enrichment in cellular pathways related to infection/inflammation and insulin signaling/resistance (Fig. 3A). The infection-related pathways essentially represented inflammatory genes, such as IL6, TNFRSF1A, and NFkB. IL17 signaling has recently

**Fig. 2.** Adipose RND3 mRNA correlates with insulin resistance and adipocyte size. A. RND3 mRNA was measured by microarrays (Illumina BeadChips) in subcutaneous and omental adipocytes and correlated with HOMA2-IR and TAG/HDL ratio (Cohort 2). B. RND3 mRNA was measured in subcutaneous adipose tissue by Affymetrix microarrays and correlated with BMI, HOMA2-IR and adipocyte number and size (Cohort 3). The Pearson correlation coefficient r and p-value are shown. Clinical data were missing for some patients in Cohort 2. BMI, body mass index; HDL, high-density lipoprotein cholesterol; HOMA2-IR, homeostatic model assessment 2 of insulin resistance; SC, subcutaneous; TAG, triacylglycerol.
been implicated in chronic inflammation in adipose tissue [36], in part dependent on signals from adipocytes [37]. The cytosolic DNA-sensing pathway also relates to inflammation, involving conversion of GTP and ATP to cyclic GMP-AMP (cGAMP) via cGAMP synthase (cGAS) which promotes cellular senescence via interferon signaling and inflammatory gene expression [38]. The co-expression analyses also indicated a link to inflammatory stimuli increased RND3 mRNA (Fig. 3D). These data are in line with the previously reported HIF-1α-mediated transactivation of RND3 in gastric cancer cells [26].

3.3. RND3 Knockdown Affects Metabolic and Inflammatory Genes

So far the presented data are correlative in terms of downstream functions of RND3 in adipose tissue. To establish causality, we differentiated primary human adipocytes in vitro (Fig. 4A) and performed RNA sequencing with and without siRNA-mediated knockdown of RND3. Based on the expression profile of PPARG2, a master regulator of adipogenesis and adipocyte function, we performed the knockdown on day 6–8 of differentiation coinciding with peak PPARG2 levels on day 8 (Fig. 4B). Of note, RND3 mRNA was largely unaltered during...
differentiation, indicating a relatively high expression in both adipocyte progenitors and mature adipocytes (qPCR Ct values around 25–26) (Fig. 4B). For primary adipocyte cultures obtained from 6 women (BMI 28.8 ± 2.4 kg/m2, age 39.5 ± 12.6 years), principal component analysis (PCA) of the RNA sequencing data showed a clear separation of samples treated with non-targeting or RND3-targeting siRNA (Fig. 5A). A volcano plot showed that RND3 was, except for DDAH1, the most significant differentially expressed gene in cells with RND3 knockdown, and revealed a tendency of greater overall downregulation than upregulation (Fig. 5B). Although the 6 experiments showed a notable individual variation (Fig. 5C), paired analysis of controls compared to knockdown for each individual retrieved a total of 134 downregulated and 92 upregulated genes within a false discovery rate (FDR) < 0.1 cutoff (Table S1).

Pathway analysis for these transcripts showed an enrichment of upregulated genes in adipocyte lipolysis, AMPK signaling and insulin signaling, and of downregulated genes in pro-inflammatory pathways reminiscent of the in vivo co-expression analysis (Fig. 5D). Among the genes involved in lipolysis were ADCY1, IRS2, LPE and SCD, which were also linked to insulin-mediated suppression of lipolysis and AMPK signaling (Fig. 5E). Additional genes related to insulin signaling included FASN, ACACB and PCK1 associated with lipid storage (e.g., PCK1 (PEPCK) controls adipocyte glyceroneogenesis to promote intracellular esterification of FFA to glycerol [40]).

3.4. ROCK Expression Associates with Adipocyte Metabolism and Obesity

Given the role of Rho kinase (ROCK) in mediating cellular effects of RND3, with important metabolic roles in skeletal muscle [23] and adipose tissue [41,42], we further reanalyzed a published global gene expression dataset for a mouse dedifferentiated fat (DFAT) cell line treated for 4 days with and without ROCK inhibitor [43]. Significantly enriched pathways in the ROCK-inhibitor treated cells were regulation of lipolysis in adipocytes and glycerolipid metabolism (i.e., glyceride/FFA cycling [44]), along with PPAR signaling (all for upregulated genes; no enrichment was seen for downregulated genes) (Fig. 6A). The increased expression of genes in these pathways coincided with a stimulatory effect of the ROCK inhibitor on adipogenic redifferentiation in the DFAT cells [43].

Metabolic effects of the ROCK inhibitor could depend on ROCK isoform expression. To assess the relevance of the respective isoforms in adipose and other tissues, we examined Rock1 and Rock2 mRNA expression levels and their changes in metabolic tissues during diet-induced obesity and insulin resistance. Rock1 showed the highest expression in pancreatic islets, adipose tissue and liver, and Rock2 in muscle and adipose tissue (Fig. 6B). However, only adipose tissue showed significant increases in Rock1 as well as Rock2 mRNA in the obese ob/ob B6 and insulin resistant BTRB mice, particularly after 4 weeks of feeding. On the other hand, liver and muscle showed significant reductions in Rock1 mRNA, and no changes in Rock2 mRNA (Fig. 6B).

3.5. RND3 Regulates Adipocyte Lipolysis

The effect of RND3 knockdown on genes involved in adipocyte lipolysis prompted us to measure glycerol release as a direct measure of lipolysis. Lipolysis can be induced through increased levels of beta-adrenergic receptor activation and cAMP, with consequent activation of PKA which phosphorylates specific residues on HSL [12,13]. When treating developing primary human adipocytes with cell membrane-permeable cAMP analogs, we observed a 1.5-fold increase in RND3 mRNA. This effect likely occurred through the PKA pathway since the cAMP analog 8CPT-cAMP, which targets PKA as well as the alternative Epac pathway, showed no additional effect compared to the PKA-specific analog 6 MB-cAMP (Fig. 7A).

We next assayed lipolysis measured as glycerol release following RND3 knockdown in primary human adipocytes. Knockdown decreased lipolysis up to 35% depending on the cellular condition (Fig. 7B). Whereas knockdown reduced basal lipolysis only slightly, the knockdown potently attenuated CAMP-stimulated lipolysis. Conversely, treatment with ROCK inhibitor increased lipolysis about 2-fold, consistent with a positive effect of ROCK on insulin-mediated suppression of lipolysis. Surprisingly, however, the ROCK inhibitor attenuated the CAMP-stimulated lipolysis (Fig. 7B), possibly due to a saturation effect as cAMP stimulation already increased lipolysis 50-fold. We further found that RND3 knockdown inhibited lipolysis induced by isoproterenol, a catecholamine-mimicking beta-adrenergic receptor agonist that activates cAMP/PKA signaling in adipocytes. When treated with ROCK inhibitor, to “rescue” the loss of ROCK inhibition upon RND3 knockdown, the adipocytes showed increased basal as well as isoproterenol-stimulated lipolysis as expected (Fig. 7B).

PKA-mediated phosphorylation of ser650 on HSL (typically referred to as ser660 which is the equivalent residue in rat) is an important mechanism for inducing adipocyte lipolysis in vivo, through a crosstalk with AMPK signaling which also regulates ATGL activity [14]. We therefore measured the effect of RND3 knockdown and ROCK inhibitor on pHSLser650 by Western blotting, as well as on total HSL and ATGL levels. The Western blots confirmed a reduction in RND3 protein (Fig. 7C). Both knockdown and ROCK inhibitor reduced ATGL protein levels. Further, while total HSL levels were unchanged, levels of pHSLser650 were reduced (Fig. 7C), indicating that RND3 and RhoA/ROCK are required for PKA-mediated activation of lipolysis in adipocytes. Taken together, our clinical and experimental data support a
Fig. 5. RND3 knockdown affects genes involved in lipid metabolism and inflammation in primary human adipocyte cultures. Cultures from 6 people were transfected with control non-targeting siRNA (siNT) or siRNA targeting RND3 from day 6 to 8 after induction of adipocyte differentiation (48 h). Global gene expression was measured by RNA sequencing. A. Principal component analysis (PCA) depicting the effect of RND3 knockdown on global gene expression (adjusted for effects of the individuals). B. Volcano plot depicting fold changes for differentially expressed genes comparing non-targeting and RND3 siRNA. C. Heatmap showing relative expression of the 50 most up-regulated and 50 most down-regulated genes with RND3 knockdown (for genes with FDR < 0.1 in the RNA-seq dataset). 0 represents an expression of 0, and 1 represents the maximum observed expression for that gene across all samples, to which all other samples for the same row are compared. D. KEGG pathways with a significant enrichment of differentially expressed genes with RND3 knockdown (for genes with false discovery rate (FDR) < 0.1). All pathways passed FDR < 0.05 for enrichment. E. Relative expression of the genes in the enriched KEGG pathways for up-regulated genes with RND3 knockdown. Data are presented as mean ± SD. Reg., Regulation; siNT, small interfering non-targeting RNA.
novel mechanism by which RND3 promotes insulin resistance syndrome through inhibition of ROCK proteins, which alleviates an inhibitory effect on adipocyte lipolysis (Fig. 7D).

4. Discussion

In the present study we explored the potential roles of the Rho GTPase RND3 in obesity-related adipose tissue function. We revealed elevated RND3 mRNA expression in obesity, and novel correlations between adipose RND3 mRNA expression and features of insulin resistance syndrome in humans, including positive correlations with HOMA2-IR, TAG/HDL ratio and adipocyte size. These correlations were partly independent of BMI. Our experimental data further indicate that altered RND3 expression causally regulates adipose tissue function, with a particular link to pathways involved in inflammatory responses and adipocyte lipid handling.

We found that RND3 knockdown consistently reduced adipocyte lipolysis, in the basal state as well as upon stimulation of lipolysis by cAMP or beta-adrenergic receptor agonist (isoproterenol). Conversely, pharmacologic inhibition of ROCK overall increased lipolysis, consistent with a lipolytic effect of RND3-mediated ROCK inhibition which may impair insulin signaling. These data are in line with our observed association between RND3 mRNA and insulin resistance, as well as with previous studies implicating reduced skeletal muscle ROCK1 activity in systemic insulin resistance [23,45]. Further consistent with our data linking RND3 to impaired insulin signaling and lipolysis, selective inhibition of ROCK2 in 3T3-L1 adipocytes was recently shown to impair adipogenesis [42]. Furthermore, this ROCK2 inhibitor was found to promote lipid accumulation in adipose tissue macrophages (ATMs) and thereby favor macrophage polarization towards the pro-inflammatory M1 rather than the anti-inflammatory M2 type [46]. Thus, increased adipocyte lipolysis due to increased RND3 activity might promote pro-inflammatory lipid accumulation in ATMs. This effect may be further exacerbated by increased RND3 mRNA expression upon pro-inflammatory stimuli (LPS, TNFα) as we observed in primary human hASCs. These stimuli upregulate lipolytic genes partially via

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**Fig. 6.** Effect of ROCK inhibition on metabolic pathways in DFAT cells and expression of ROCK isoforms in tissues from obesity- and insulin resistance-prone mice. A. The mouse DFAT (dedifferentiated fat) cell line was treated for 4 days with and without ROCK inhibitor (Y-27632, 30 mM), as described previously [43]. Genes with a fold change >2 compared to control were subjected to KEGG pathway analysis, revealing highly significant fold enrichment of these genes in three categories relative to the count of genes in these pathways expected by chance. B. Lean and obese (ob/ob) B6 and BTBR mice were fed for 4 or 10 weeks (ad libitum). Adipose tissue (epididymal), liver, hypothalamus, soleus, gastrocnemius and pancreatic islets were analyzed (n = 5). Data are presented as mean ± SD. P-values were calculated by one-way ANOVA and Tukey's posthoc test. B6, C57BL/6 inbred mouse strain; BTBR, Black and Tan BRachyury mouse strain; Ob, obese; Ob/ob, obese leptin-deficient. * p < .05; ** p < .01; *** p < .001.
NF-κB transcriptional activity [47] as well as cAMP [48, 49]. Adipocyte RND3 may therefore be part of a vicious cycle of insulin resistance, inflammation and adipocyte lipolysis that manifests upon chronic overfeeding.

The comparison of ROCK expression across tissues points to an important role for both ROCK isoforms in adipose tissue, which showed increased expression in mouse models of diet-induced obesity and insulin resistance. Despite higher expression of Rock2 in leg muscle, we only observed altered Rock2 expression in adipose tissue. Furthermore, the change in Rock1 expression was opposite in adipose tissue compared to other metabolic tissues. These data support specific roles for the ROCK proteins in adipocytes. The increase in ROCK expression specifically in adipose tissue might reflect a transcriptional feedback mechanism that attenuates the loss of ROCK activity upon increasing insulin resistance. It should be noted, however, that when knocking out the ROCK1 isoform selectively in adipocytes, Lee et al. found enhanced signaling through the insulin receptor, although the isoform-specific loss of ROCK1 only modestly affected whole-body insulin sensitivity [41]. It could be that the increased adipocyte lipolysis observed upon RND3 knockdown and ROCK inhibition involved enhanced insulin signaling, given the co-expression we observed upon RND3 knockdown of genes involved in lipolysis and insulin signaling, and since prolonged insulin exposure has been found to enhance cAMP- and isoproterenol-stimulated lipolysis [50]. However, the precise mechanisms that mediate the effect of RND3 on HSL/ATGL levels and adipocyte lipolysis need to be further delineated. The RNA-sequencing data suggest that AMPK signaling, which is elevated in conditions associated with lipolysis such as fasting, could also be involved. Further, we cannot rule out effects of RND3 also independent of ROCK signaling, as in the regulation of cell cycle progression [22].

Importantly, whole-body insulin sensitivity can improve upon partial inhibition of lipolysis [6, 16], supporting that RND3-mediated stimulation of lipolysis may be a causal mechanism that leads to insulin resistance, and making RND3 a potential therapeutic target to prevent the development of insulin resistance, inflammation and associated metabolic diseases. We further found a positive correlation between RND3 mRNA and adipocyte size. Adipocyte hypertrophy is a hallmark of adipose tissue inflammation in patients with insulin resistance independent of obesity [4, 51]. Moreover, adipocyte hyper trophy may cause adipose and systemic insulin resistance also in the absence of adipose inflammation [52]. Despite their increased lipid accumulation, hypertrophic adipocytes are characterized by elevated rates of lipolysis, which may contribute to increased circulating FFA concentrations, ectopic lipid accumulation, and systemic insulin resistance [9, 53].

The inverse relationship we found between RND3 in adipocytes and a PPAR-related gene signature suggests a mechanism by which RND3 promotes insulin resistance, since adipocyte PPAR activity promotes whole-body insulin sensitivity, epitomized by pharmacologic treatment with PPARγ-specific ligands such as rosiglitazone. Rosiglitazone-mediated PPARγ activation promotes glycero nogenesis in subcutaneous adipocytes to favor TAG synthesis over lipolysis [54], in a subtle balance between lipid storage and release with implications for systemic insulin resistance and metabolic disease risk [9]. Accordingly, we previously demonstrated that adipocytes of people who are susceptible to insulin resistance and T2D, as a result of carrying a T2D risk allele in the PPARγ locus, have decreased glycero nogenesis (pyruvate incorporation into TAG) and increased lipolysis (FFA release) [55]. Consistently, dose-dependent restoration of insulin resistance with rosiglitazone involves decreased levels of CAMP and inhibition of lipolysis [56]. It should be noted that elevated circulating FFA levels in T2D may also result from de novo lipogenesis and impaired lipid handling independent of lipolysis [17].

It is further possible that RND3 at least partly affected adipocyte lipolysis by modulating actin stress fibers and cell structure, as a primary function of RND3 is to serve as an endogenous antagonist of RhoA/ROCK signaling and thereby modulate actin cytoskeleton dynamics [21]. Effects of RhoA/ROCK signaling on cytoskeleton dynamics has been implicated in adipocyte function [43]. Another small GTPase which regulates actin cytoskeleton, membrane receptor trafficking and endocytosis, ADP-ribosylation factor 6 (Arf6), has been found to modulate lipolysis in response to beta-adrenergic (but not cAMP) stimulation, which may involve adrenergic receptor trafficking [57]. In line with our findings for RND3 knockdown, Arf6 knockdown in 3T3-L1 adipocytes reduced isoproterenol-stimulated lipolysis. Future studies should explore the possible role of altered

Fig. 7. Knockdown of RND3 reduces cAMP- and isoproterenol-stimulated lipolysis. Primary human adipose stromal cells or 3G25 cells were differentiated in vitro. A. Human adipose stromal cells differentiated towards adipocytes for 3 days were treated with cAMP analogs (6 Mb-cAMP or 8CPT-cAMP) for 24 h. RND3 mRNA was measured by qPCR (calculated relative to PO8). Data are presented as mean ± SD. B. hASCs were transfected at day 8 of differentiation with siRNAs targeting RND3. On the day of analysis, the cells were incubated for 3 h in DMEM-F:12 medium supplemented with 20 g/L of BSA and 1 mM of dibutyryl cAMP (dbcAMP) or 1 μM ROCK inhibitor (ROCKi). Results are based on three independent experiments with each condition performed at least in triplicates. C. Differentiated SGbSS cells (day 15) were transfected with control or RND3 siRNA for 72 h, and treated with 10 μM ROCK inhibitor for 24 h. Protein samples were analyzed by SDS-PAGE and immunoblotting (20 μg protein for the p-ser650 HSL and ATGL blots). Membranes were blocked by 5% BSA for 1 h at room temperature, followed by repeated washing in 1xTBS twice, overnight exposure to the primary antibody in buffer with 5% BSA at 4°C, repeated washing, exposure to the secondary antibody (anti-rabbit 1:10000 or anti-mouse 1:5000) in 5% BSA for 30 min at room temperature, repeated washing, and detection by ChemiDoc MP imager (BioRad). D. Proposed mechanistic model for the contribution of RND3 to insulin resistance syndrome, mediated via inhibition of ROCK signaling, supported expression and activation of rate-limiting lipolytic enzymes HSL and ATGL, and resultant augmentation of lipolysis in adipocytes, cAMP, cyclic adenosine monophosphate; ROCKi, ROCK inhibitor; siRNA, small interfering RNA. * p < .05; ** p < .01.
actin cytoskeleton and receptor trafficking in mediating the RND3-dependent regulation of lipolysis.

Our study has strengths as well as limitations. A major strength of our study is the analyses of adipose gene expression and clinical data in several human cohorts, and experimental and functional assays performed in primary human adipocyte cultures based on a systematic whole-genome screen. On the other hand, the in vivo human expression data were correlative and do not establish causality, and we cannot necessarily extrapolate the in vitro data to an organismal context. For example, although we found a causal effect of RND3 perturbation on adipocyte lipolysis in cultured primary human adipocytes, e.g., in the context of CAMP signaling which partly mediates lipolytic effects of pro-inflammatory stimuli [48,49], these cultures do not necessarily reflect the context of cellular stress associated with obesity and insulin resistance in vivo. Thus, our data encourage future studies that perturb adipocyte Rnd3 in vivo, such as by adipocyte-specific conditional knockout in a mouse model of diet-induced obesity and insulin resistance. Finally, although we used ROCK inhibitor to mimic increased endogenous RND3 activity, RND3 overexpression in primary human adipocytes could further inform on the dynamics of lipolysis regulation at different RND3 levels, also in the context of adipose tissue stress related to insulin resistance. In primary in vitro differentiated adipocytes, such studies must be carefully balanced to ensure physiological expression levels and avoid non-specific toxic effects.

5. Conclusion

In conclusion, we here uncovered a differential expression of adipose RND3 in obesity and insulin resistance, and a causal role for RND3 in adipocyte lipolysis. Mechanistically, RND3 knockdown reduced protein levels of both ATGL and ser650-phosphorylated HSL, attenuating the stimulatory effects of isoproterenol and CAMP signaling on lipolysis. These clinical and experimental data together highlight RND3 as a novel potential target for therapeutic intervention to improve adipocyte lipid handling and ameliorate obesity-related insulin resistance.

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Author Contributions

TR, GM and SND conceived and designed the study and analyzed the data. TR, AK, ZF and SND performed the experiments. TR, GM, JVS, AK and SND analyzed the clinical data. TR, GM and SND conceived and designed the study and analyzed the data. TR, AK, ZF and SND performed the experiments. SND, GM, JVS, TR, AK, ZF and SND analyzed the clinical data. All authors declare no conflict of interest.

Declaration of Competing Interest

All authors declare no conflict of interest.

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