Adiponectin release and insulin receptor targeting share trans-Golgi-dependent endosomal trafficking routes

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ABSTRACT

Objective: Intracellular vesicle trafficking maintains cellular structures and functions. The assembly of cargo-laden vesicles at the trans-Golgi network is initiated by the ARF family of small GTPases. Here, we demonstrate the role of the trans-Golgi localized monomeric GTPase ARFRP1 in endosomal-mediated vesicle trafficking of mature adipocytes.

Methods: Control (Arfrp1fl/fl) and inducible fat-specific Arfrp1 knockout (Arfrp1iAT-/-) mice were metabolically characterized. In vitro experiments on mature 3T3-L1 cells and primary mouse adipocytes were conducted to validate the impact of ARFRP1 on localization of adiponectin and the insulin receptor. Finally, secretion and transferrin-based uptake and recycling assays were performed with HeLa and HeLa M-C1 cells.

Results: We identified the ARFRP1-based sorting machinery to be involved in vesicle trafficking relying on the endosomal compartment for cell surface delivery. Secretion of adiponectin from fat depots was selectively reduced in Arfrp1iAT-/- mice, and Arfrp1-depleted 3T3-L1 adipocytes revealed an accumulation of adiponectin in Rab11-positive endosomes. Plasma adiponectin deficiency of Arfrp1iAT-/- mice resulted in deteriorated hepatic insulin sensitivity, increased gluconeogenesis and elevated fasting blood glucose levels. Additionally, the insulin receptor, undergoing endocytic recycling after ligand binding, was less abundant at the plasma membrane of adipocytes lacking Arfrp1. This had detrimental effects on adipose insulin signaling, followed by insufficient suppression of basal lipolytic activity and impaired adipose tissue expansion.

Conclusions: Our findings suggest that adiponectin secretion and insulin receptor surface targeting utilize the same post-Golgi trafficking pathways that are essential for an appropriate systemic insulin sensitivity and glucose homeostasis.

Keywords Adiponectin; ARFRP1; Exocytosis; Insulin receptor; trans-Golgi

1. INTRODUCTION

An appropriate adipose tissue function is a major determinant of metabolic health. Beside storage and supply of energy, the adipose tissue possesses a secretory capacity releasing a variety of bioactive molecules into the circulation, such as adipokines, cytokines, and lipid metabolites. To meet these secretory requirements, adipocytes crucially rely on a sophisticated vesicle trafficking machinery. However, despite that there is substantial knowledge about physiological effects of secreted factors, such as leptin and adiponectin, precise intracellular trafficking pathways and particular molecular components mediating the targeting and release of bioactive molecules are still largely elusive. Within the classical secretory pathway, transmembrane and soluble cargos travel via the endoplasmic reticulum (ER) and Golgi apparatus en route to their final destinations [1]. However, at a post-Golgi level, trafficking routes can diverge. In this context, the trans-Golgi network (TGN) is conventionally regarded as the main cargo sorting station; together with a broad range of accessory proteins, the TGN regulates accurate packaging of cargo into the right transport vesicle and delivery along correct trafficking routes. The assembly of cargo-laden vesicles at the TGN is initiated by the ARF family of small GTPases consisting of ARF, SAR (secretion-associated and Ras-related), and ARL (ARF-like) proteins [2]. Mammalian cells possess 6 ARF proteins and more than 20 ARF-like proteins, whose intracellular roles are poorly understood [3]. Among them, ARFRP1 (ADP-ribosylation factor-related protein 1), which localizes in the activated GTP-bound form to the TGN, has been functionally implicated in vesicle trafficking of VSVG (vesicular stomatitis virus G) [4,5], E-cadherin [6], Vangl2 (vang-like 2)
more, conditional adipocyte-specific deletion of Arfp1 resulted in a severe lipodystrophic phenotype of newborn mice underlining the pivotal role of ARFRP1 for the development of functional adipose tissue depots [10]. To explore the impact of ARFRP1 as part of the vesicle trafficking machinery on the secretory capacity of mature adipocytes, we generated a mouse model with an inducible fat-specific disruption of the Arfp1 gene (Arfp1flAT−/−). Here, we demonstrate that the loss of Arfp1 from differentiated adipocytes diminishes adiponectin secretion and plasma membrane localization of the insulin receptor associated with detrimental effects on adipocyte metabolism and glucose homeostasis. These findings were attributed to defective endosomal-mediated exocytosis that was monitored in vitro following Arfp1 suppression.

2. MATERIAL AND METHODS

2.1. Animals
In order to generate inducible adipocyte-specific Arfp1 knockout animals on a C57Bl/6J background, transgenic mice carrying floxed Arfp1 alleles (Arfp1fllox/lox, N10 generation) [6] were crossed with mice heterozygously expressing the CreERT2-transgene [11] under the control of the adipocyte-specific adiponectin promoter (AdipoqCreER2, kindly provided by Prof. Dr. Stefan Oeffermans, MPI, Bad Nauheim) [12]. Mice were kept at a temperature of 22 ± 2 °C with a 12:12 h light–dark cycle and ad libitum access to drinking water and phytoestrogen-reduced standard diet (V1554-000 R/M–H, Sniff). At 5 weeks of age, male Arfp1flAT−/− and Arfp1fllox/lox mice received tamoxifen (1 mg/d, Sigma Aldrich) by oral gavage for 5 consecutive days continued by feeding a tamoxifen-containing, phytoestrogen-reduced standard diet (400 mg/kg tamoxifen D.CRTAM400.R1, LAS-vendi GmbH) until the end of the study (7 or 10 weeks of age). This resulted in adipocyte-specific disruption of the Arfp1 gene in Arfp1flAT−/− mice. All animal experiments were approved by the ethics committee of the State Office of Environment, Health and Consumer Protection (Federal State of Brandenburg, Germany).

2.2. Body composition, blood glucose, pyruvate tolerance test (PTT), and insulin response
Body composition of inducible adipocyte-specific Arfp1 knockout mice (Arfp1flAT−/−) and control littermates (Arfp1fllox/lox) was determined weekly by nuclear magnetic resonance (EchoMRITM-100H, EchoMRI) by oral gavage for 5 consecutive days continued by feeding a tamoxifen-containing, phytoestrogen-reduced standard diet (400 mg/kg tamoxifen D.CRTAM400.R1, LAS-vendi GmbH) until the end of the study (7 or 10 weeks of age). This resulted in adipocyte-specific disruption of the Arfp1 gene in Arfp1flAT−/− mice. All animal experiments were approved by the ethics committee of the State Office of Environment, Health and Consumer Protection (Federal State of Brandenburg, Germany).

2.3. Ex vivo secretion and lipolysis assay
Gonadal (gon) and subcutaneous (sc) white adipose tissue (WAT) depots were removed from 7-week-old Arfp1flAT−/− and Arfp1fllox/lox mice, rinsed in prewarmed PBS, minced into small pieces (~10 mg), and incubated in phenolred-free DMEM (PAN-Biotech) supplemented with 4.5 g/l glucose, 25 mmol/l HEPES, 1 mmol/l sodium pyruvate, 4 mmol/l glutamine, 1% penicillin/streptomycin, and 2% fatty acid-free BSA at 37 °C and 5% CO2 for 24 h. Thereafter, supernatants were collected, centrifuged (500 g for 5 min), and analyzed for adiponectin and leptin concentration. To measure extracellular lipolysis rates, gonWAT explants were incubated in the above-mentioned medium in the absence (basal lipolysis) or presence (stimulated lipolysis) of 10 μmol/l isoproterenol (Sigma—Aldrich) together with 1 μmol/l insulin (Roche) when indicated for 2 h before detection of non-esterified fatty acids (NEFA) in the supernatant. Fat explants were washed, collected and lyzed for protein measurement. Adiponectin and NEFA levels were normalized to respective fat explant protein concentration.

2.4. Plasma and supernatant analyses
Designated adipokines were quantitatively assessed in plasma and supernatant of fat explants using respective ELISA kits (DY1119, MOB00, DY5430-05, DY1069, MWSP10, DY954, R&D Systems GmbH) following the manufacturer’s instructions. Triglyceride, glycerol (TR0100, Sigma—Aldrich), and NEFA (NEFA-HR/2, Wako Chemicals GmbH) levels were measured in plasma and fat explant supernatant according to manufacturer’s protocol.

2.5. Subcellular fractionation of primary and 3T3-L1 adipocytes
Subcellular fractionation of isolated primary adipocytes from gonWAT of 7-week-old Arfp1flAT−/− and Arfp1fllox/lox mice (5–6 mice pooled per genotype) as well as 3T3-L1 adipocytes transfected with nt- (non-targeting) or Arfp1-siRNA was performed as described before [13].

2.6. Protein extraction and western blotting
Tissue lysates were prepared using RIPA buffer [50 mM Tris—HCl (pH 7.2), 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% Triton X-100, 2 mM Na3VO4] supplemented with phosphatase inhibitor (PhosSTOP, Roche) and protease inhibitor (cComplete, Roche). Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer’s protocol. Western blot analysis of lysates and fractions from mouse tissue or 3T3-L1 adipocytes was performed as described previously [14]. The following primary antibodies were applied overnight at 4 °C: ARFRP1 (1:500; Abcam, ab108199), insulin receptor β subunit (IRβ) (1:500; Merck Millipore, GR36), INS-1 (1:500; Cell Signaling, 3407), IRS-2 (1:500; Merck Millipore, MABS15), AKT (1:1,000; Cell Signaling, 9727), pAKT (Ser473; 1:1,000; Cell Signaling, 9727), AMPlkα (1:1,000; Cell Signaling, 2532), AMPlkβ (Thr172; 1:1,000; Cell Signaling, 2532), tFOXO (1:1,000; Cell Signaling, 2880S), pFOXO (1:1,000; Cell Signaling 9464S), adiponectin (1:500; Abcam, ab3455), calnexin (1:2,000; Abcam, ab22595), Rab11 (1:1,000; Thermo Fisher Scientific, 71—5300), α-tubulin (1:500; Sigma—Aldrich, T6199), and GAPDH (1:10,000; Ambion, AM4300). For non-denaturing western blot analysis of adiponectin isoforms, equal volumes of murine plasma samples were pre-diluted (1:30) and mixed with 2× adiponectin sample buffer [3% SDS, 20% Glycerol, 50 mM Tris, 0.02% Bromophenol blue] before incubation at room temperature for 1 h and separation in a precast gradient gel (4—15% Ready Gel Tris—HCl Gel, Bio-Rad). Corresponding HRP-conjugated secondary antibodies (Dianova) were applied (1:20,000) for 1 h at room temperature before targeted proteins were visualized using Amersham ECL Western Blotting Detection kit (GE Healthcare).

2.7. Histology
Adipose tissue depots were fixed in 4% formaldehyde and embedded in paraffin. Sections (2 μm) were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) using a standard protocol before dehydrated and mounted. Microscopic analysis was performed...
with the Keyence BZ-9000 Fluorescence Microscope and corresponding BZ-II Analyzer software (Keyence International). Adipocyte area of 3 histological images per animal and 3–5 mice per genotype and age were assessed by applying an automated image analysis software (WimAdipose, Wimsas GmbH).

2.8. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from mouse tissues was extracted using RNeasy Mini Kit (QIAGEN) according to manufacturer’s instructions. RNA samples were reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega). Gene expression levels were detected applying TaqMan Gene Expression Assays (Atrfp1 Mm01220415_g1, Adipq Mm00456425_m1; Gαbpc Mm00839363_m1, Pck1 Mm01247058_m1, Eef2 Mm01171434_g1) by using the LightCycler® 480 II/384 (Roche). Target gene expression was normalized to Eef2 used as endogenous control.

2.9. Cell culture and si-RNA transfection

All cell lines were kept at 37 °C and 5% CO2. 3T3-L1 cells (ATTC® CL-173TM) were cultured in IMDM (4.5 g/l glucose, PAN-Biotech) supplemented with 10% newborn calf serum (NCS, PAN-Biotech). Differentiation of confluent cells was induced by applying IMDM including 10% fetal bovine serum (FBS, PAN-Biotech), 1.2 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 0.25 μM dexemethasone (Sigma—Aldrich) and 2 μM rosiglitazone (Sigma—Aldrich). At day 3 and day 4 medium was replaced by IMDM containing 10% FBS and 1.2 μg/ml insulin. Transfection with indicated siRNAs was performed by electroporation at day 6 as previously described [10], and cells were cultured for further 96 h before used in different experimental setups. HeLa (ATCC®) and HeLa M-C1 cells were cultured in DMEM (4.5 g/l glucose, Life Technologies) containing 10% FCS and 50 μg/ml penicillin/streptomycin. When reaching 40% confluency, cells were transfected with designated siRNAs by using Oligofectamine (LifeTechnologies) according to manufacturer’s instructions, re-seeded on matrigel-coated (BD-Bioscience) coverslips the following day, and cultured for further 24 h before used in immunofluorescence-based assays.

2.10. HeLa M-C1 secretion assay

For monitoring constitutive secretion, transfected HeLa M-C1 cells [15] were washed with PBS and treated with 1 μM D/O doulisolubilizer (Ciontech) diluted in Hank’s Balanced Salt Solution (HBSS, Gibco) to initiate secretion of the reporter. At the indicated time points, cells were placed on ice, washed twice with ice-cold PBS including 10 mM MgCl2, and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. GFP intensity was quantified per cell, and all time points were normalized to respective 0 min reflecting whole amount of reporter located within the ER.

2.11. Transferrin recycling

Transfected HeLa cells and 3T3-L1 adipocytes were serum-starved for 1 h and incubated with 15 μg/ml Alexa568-labeled transferrin (Alexa568-Tf, Life Technologies) at 37 °C for 30 min to saturate receptor-ligand uptake and recycling. After being washed with ice cold phosphate-buffered saline (PBS) containing 10 mM MgCl2, cells were acid washed at pH 5.3 (0.1 M Na-acetate, 0.5 M NaCl) for 1 min on ice to remove surface-bound Tf (referred to 0 min), followed by washing with ice cold PBS/MgCl2 and incubation with 1 mg/ml unlabeled Tf (Sigma—Aldrich) at 37 °C for 20 min to allow receptor-ligand exocytosis. Cells were fixed after 0 and 20 min with 4% PFA for 45 min at room temperature. Alexa568-Tf intensity was quantified per cell and the relative amount of released Tf was calculated as a measure of Tf—TfR exocytosis. For quantification of subcellular Tf—TfR localization, cells incubated with Alexa568-Tf at 37 °C for 30 min were classified into the following groups: overlapping Tf—TfR signals distributed throughout the entire cell (whole cell), primarily in the perinuclear region (perinuclear) or at the cell periphery (tip). Counted cells per classification were expressed as percentage of total number of cells.

2.12. Transferrin surface labeling and uptake

Transfected HeLa cells and 3T3-L1 adipocytes were serum-starved for 2 h and incubated with 15 μg/ml Alexa568-Tf for 50 min at 4 °C to block endocytosis. One set of cells was washed with ice cold PBS/MgCl2 and fixed with 4% PFA for 45 min at room temperature to determine surface-bound Alexa568-Tf as a measure of TfR surface localization. Another set of cells was transferred to 37 °C for 5 min allowing Tf—TfR endocytosis, followed by washing with PBS/MgCl2 and fixation with 4% PFA for 45 min at room temperature. Alexa568-Tf intensity was quantified per cell and the rate of endocytosis was calculated by the ratio of Tf uptake to Tf surface.

2.13. Immunocytochemistry

For immunocytochemical stainings of 3T3-L1 adipocytes, cells were serum-starved for 4–6 h, fixed with 4% PFA for 10 min, rinsed with PBS, washed with 50 mM NH4Cl (Roth) and subsequently permeabilized for 6 min with 0.2% saponin (Sigma—Aldrich). After being washed with 0.02% saponin, cells were incubated for 30 min in blocking solution (2% BSA (Sigma—Aldrich) in 0.02% saponin) before application of primary antibodies diluted in blocking solution overnight at 4 °C. The following primary antibodies were used: ARFRP1 (1:100; Abcam, ab108199), insulin receptor β subunit (1:50; Merck Millipore, GR36), Rab11 (1:50; Thermo Fisher Scientific, 71-5300), γ-adaptin (1:100; BD Transduction Lab, 610385), LAMP1 (1:50; Abcam, [H4A3] ab25630), lepitin (1:1000, anti-rabbit, self-made), adiponectin (1:100; Abcam, ab25635, ab22554), caveolin-1 (1:100; BD Transduction Lab, 610059). Thereafter, cells were incubated with corresponding AlexaFluor-labeled secondary antibodies (1:200; Thermo Fisher Scientific) and DAPI (1:1,000,000; Roche) diluted in blocking solution for 1 h at room temperature, followed by washing with 0.02% saponin and mounting using Fluorescence Mounting Medium (Dako). Imaging and quantification was performed with the Leica TCS SP8 X Laser Scanning Confocal Microscope (Leica Microsystems) and the corresponding image analysis software (Leica Application Suite X version 1.81). Staining of fixed HeLa and HeLa M-C1 cells was performed as described in [16] using the following primary antibodies: ARFRP1 (1:200; Abcam, ab108199), transferrin receptor (1:300; Life Technologies, 13—6800), GM130 (1:100; BD Transduction Lab, 610822) and AlexaFluor-labeled secondary antibodies (1:200; Life Technologies). Cells were mounted using Immu-Mount (Thermo Fisher Scientific) containing 10 μg/ml DAPI (Life Technologies). Stainings were analyzed and quantified using a spinning disc confocal microscope (Ultraview ERS, Perkin Elmer) with Velocity imaging software (Improvision, Perkin Elmer).

2.14. Statistical analysis

All data are reported as mean ± SEM of at least three different experiments, except when otherwise indicated. To compare differences between two groups, paired or unpaired Student’s t-test or were performed. Differences between two groups over the time were evaluated using two-way ANOVA with Bonferroni’s correction for multiple comparisons. Statistical analysis was performed with Prism 5 (GraphPad). Significance was accepted at *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
3. RESULTS

3.1. Adipocyte-specific deletion of Arfrp1 selectively reduces adiponectin and adipasin secretion

As our previous studies have shown that the monomeric GTPase ARFRP1 regulates intracellular trafficking of certain cargo molecules and is indispensable for proper adipose tissue development, we aimed to clarify the role of ARFRP1 on secretory processes in mature adipocytes. We specifically deleted Arfrp1 in adipocytes by tamoxifen treatment of 5-week-old Arfrp1<sup>fl<sup>ox/lox</sup></sup> × AdipoqCreER<sup>T2</sup> offspring mice. As shown in Figure S1 tamoxifen treatment resulted in a specific suppression of Arfrp1 expression in adipose tissues, other organs were not affected. Two weeks following initiation of gene deletion, we screened for the abundance of different adipokines in the plasma of 7-week-old control (Arfrp1<sup>fl<sup>ox/ox</sup></sup>) and inducible adipocyte-specific Arfrp1 knockout (Arfrp1<sup>AT<sup>−/−</sup></sup>) mice. Levels of adiponectin as well as adipasin were significantly reduced in the circulation of mice lacking Arfrp1 in adipose tissue deposits, whereas other adipokines such as leptin and resistin were not affected (Figure 1A). As multimodalization of adiponectin is described to be important for its secretion [17], we assessed the three multimeric forms of adiponectin by non-denaturing western blot analysis and observed similarly decreased abundance of all three isoforms in the plasma of Arfrp1<sup>AT<sup>−/−</sup></sup> compared to control mice (Figure 1B). Intriguingly, adiponectin transcript levels (Adipoq) determined in gonadal and subcutaneous white adipose tissue (gonWAT, scWAT) and in brown adipose tissue (BAT) were not altered between the genotypes (Figure 1C), indicating that ARFRP1 acts on the level of adiponectin released from fat explants of mice and control littermates. Consistent with in vivo results, adiponectin released from fat explants of Arfrp1<sup>AT<sup>−/−</sup></sup> animals was significantly lower than from those of control mice (gonWAT 55% and scWAT 59% of controls) (Figure 1D), whereas leptin release was not different between the genotypes (Figure 1E). Thus, these data reveal that ARFRP1 is specifically required for an appropriate adiponectin secretion.

3.2. Loss of Arfrp1 leads to endosomal accumulation of adiponectin

In order to define the subcellular distribution of adiponectin, isolated adipocytes from gonWAT of 7-week-old Arfrp1<sup>fl<sup>ox/ox</sup></sup> and Arfrp1<sup>AT<sup>−/−</sup></sup> mice as well as 3T3-L1 adipocytes transfecting with nt (non-targeting)- or Arfrp1-siRNA were fractionated via differential centrifugation into subcellular compartments. Purify of the different fractions was assessed by respective marker proteins, e.g. SNAP23 for plasma membranes (Figure S2). In accordance with the defective secretion of adiponectin, it accumulated in the HDM fraction, containing ER (calnexin, IRAP, Figure. S2) and endosomal (Rab11) membranes, of primary and 3T3-L1 adipocytes deficient of Arfrp1 (Figure 2A–D) indicating either ER or endosomal sequestration of the adipokine. Further characterization by immunocytochemical co-stainings of 3T3-L1 adipocytes revealed that intracellular adiponectin was largely immunopositive for recycling endosomal Rab11, an effect that was even more pronounced following Arfrp1 suppression as evaluated by Pearson’s correlation coefficient (Figure 2E,F). Overlap with lysosomal LAMP1 and the TGN marker γ-adaptin was limited (Figure 2E). An intracellular accumulation of leptin after depletion of Arfrp1 in 3T3-L1 cells by specific siRNA treatment was not observed (Figure S3). Taken together, these results suggest that defective cargo exit from endosomes may retard adiponectin release.

3.3. Loss of Arfrp1 specifically impairs endosomal-mediated trafficking

Since adiponectin has been described to exit the cell via constitutive and regulated pathways involving the endosomal compartment [18],
functionality of both secretory routes was analyzed in the presence and absence of ARFRP1. To trace endocytic and recycling processes, the transferrin receptor (TfR) and its ligand transferrin (Tf), were monitored in *nt* (non-targeting)- and *Arfrp1*-siRNA transfected HeLa cells. The TfR is internalized after Tf binding at the cell surface and delivered to early endosomes. Thereafter, the Tf-TfR-complex recycles back to the plasma membrane either directly from early endosomes (rapid recycling) or via recycling endosomes (slow recycling) [19]. To dissect ARFRP1 function in this context, transfected HeLa cells were incubated with Alexa568-labeled Tf at 37°C for 30 min to allow receptor-ligand

**Figure 2: Endosomal accumulation of adiponectin in adipocytes depleted of Arfrp1.** (A, C) Abundance of adiponectin in subcellular fractions of isolated gonadal adipocytes (gonWAT) from 7-week-old *Arfrp1*+/−*−/−* (*−/−*) and *Arfrp*+/+/+ mice (*+/+*) (A) and 3T3-L1 adipocytes transfected with *nt* (non-targeting)- or *Arfrp1*-siRNA (C). (B, D) Signal intensities were quantified by densitometric analysis and expressed as fold of control (*Arfrp1*+/−*−/−*, *si-nt*) set to 1 (n = 3 independent fractionations). Homog, homogenate; HDM, high-density microsomes; LDM, low-density microsomes; PM, plasma membrane. (D) Transfected 3T3-L1 adipocytes were co-immunostained for adiponectin (red) with either Rab11, γ-adaptin or LAMP1 (green). Scale bar: 10 μm; magnification 5 μm. (F) Quantification of co-localization was calculated by Pearson’s correlation coefficient with at least 10−15 images analyzed per condition. Values are presented as mean ± SEM, *P* < 0.05 by unpaired Student’s *t*-test.
uptake and recycling. In Arfp1-depleted cells endocytosed Tf–TIR markedly accumulated in close proximity to the plasma membrane, primarily around the cellular tips, whereas control cells displayed Tf–TIR signals throughout the entire cell or concentrated in the perinuclear region as quantified from immunostainings (Figure 3A,B). This result strongly pointed towards an impaired cargo exocytosis from endosomes when ARFRP1 is absent.

In order to monitor Tf–TIR exocytosis, we incubated transfected HeLa cells with Alexa568-Tf at 37 °C for 30 min, removed surface-bound Tf (referred to 0 min) and chased the receptor-ligand release for 20 min. At time point 0 min, endocytosed Tf was markedly lower in Arfp1-depleted cells. However, after 20 min, Tf largely remained intracellularly, whereas most of the Tf was successfully exocytosed from control cells (Figure 3C). Quantification of the relative amount of released Tf (44% vs. 18%) confirmed a significantly impaired exocytosis of Tf–TIR-containing recycling vesicles from Arfp1-knockdown compared to control cells (Figure 3D).

To test whether this results in reduced Tf re-exposure at the plasma membrane, we additionally examined cell surface localization and endocytosis of the TfR and its ligand. Incubation of transfected HeLa cells with Alexa568-conjugated Tf at 4 °C resulted in Tf binding to cell surface-localized TfR (Tf surface), followed by incubation at 37 °C for 5 min allowing clathrin-mediated endocytosis of the Tf–TIR-complex (Tf uptake). In fact, cell surface exposure of the TfR was strikingly reduced by around 45% in cells deficient of ARFRP1 (Figure 3E,F).

Accordingly, Tf uptake was markedly lower (44%) upon Arfp1 suppression. However, the rate of Tf–TIR endocytosis, calculated by the ratio of Tf uptake to Tf surface, was unaltered (Figure 3F), substantiating the notion that ARFRP1 affects endosomal trafficking at the level of exocytosis rather than being required for endosomal internalization. We next analyzed Tf uptake and recycling in 3T3-L1 adipocytes in which expression of Arfp1 was suppressed by the specific si-RNA. As demonstrated in Figure S4, the release of Tf in si-Arfp1 transfected 3T3-L1 cells is reduced which indicates an impaired exocytotic capacity (Figure S4 A, B). Similar to results obtained in HeLa cells, the relative uptake rate was not affected upon Arfp1 knockdown (Figure S4 D, E).

As adiponectin was also reported to exit the cell constitutively, we evaluated anterograde cargo trafficking via the classical constitutive secretory pathway by applying a HeLa M-C1-based secretion assay [15]. This cell line stably expresses a GFP-tagged reporter construct secretory pathway by applying a HeLa M-C1-based secretion assay [15]. This cell line stably expresses a GFP-tagged reporter construct. Constructing large aggregates, which are trapped within the ER under basal conditions. After the addition of a specific ligand (D/D solubilizer), aggregates are dissolved, allowing the reporter to be transported through the classical secretory pathway in order to be released from cells which was monitored by immunofluorescence microscopy. Although post-Golgi trafficking appeared to be delayed, constitutive secretion in general was intact in Arfp1-depleted HeLa M-C1 cells (Figure S5). Taken together, these data demonstrate that ARFRP1 is required for efficient exocytosis from endosome-derived recycling vesicles, thereby potentially promoting cell surface delivery of certain cargo released via the endosomal system such as adiponectin.

3.4. Lack of Arfp1 reduces cell surface localization of the insulin receptor

Similar to TLR, the insulin receptor is a transmembrane receptor partially undergoing endocytic recycling following ligand binding and internalization [20,21]. As we detected lower cell surface exposure of the TfR in response to Arfp1 suppression, we evaluated the localization of the insulin receptor in adipocytes of 7-week-old Arfp1AT–/– and control mice (2 weeks of tamoxifen treatment). Notably, the amount of insulin receptor analyzed in plasma membrane fractions of isolated primary adipocytes from Arfp1AT–/– mice was significantly reduced by about 50% compared to plasma membranes of control littermates (Figure 4A,B). At the same time, total insulin receptor levels measured in gonWAT lysates were unaffected (Figure 4C,D). However, following longer Arfp1 deletion, at the age of 10 weeks (5 weeks of tamoxifen treatment), the mature receptor was markedly reduced in gonWAT of Arfp1AT–/– animals while pro-insulin receptor levels showed no difference between the genotypes, suggesting that insulin receptor mis-targeting was followed by its accelerated degradation rather than resulting from transcriptional downregulation (Figure 4C,D). We confirmed the in vivo data by analyzing the subcellular distribution of the insulin receptor in differentiated 3T3-L1 adipocytes. In agreement with membrane fractions of primary adipocytes, immunocytochemical co-stainings revealed less cell surface localization of the insulin receptor in response to Arfp1 suppression (Figure 4E). Accordingly, co-localization of the insulin receptor with the plasma membrane marker caveolin-1, evaluated by Pearson’s correlation coefficient, was significantly decreased in Arfp1-depleted cells (Figure 4F). Taken together, we demonstrate that in addition to the TfR in HeLa cells, the insulin receptor in adipocytes was markedly reduced at the plasma membrane, suggesting that ARFRP1 promotes efficient receptor recycling back to the cell surface by regulating endosomal-mediated exocytosis.

3.5. Adipocyte-specific deletion of Arfp1 affects insulin response, lipolytic activity, and expandability of adipose tissue

As we found that ARFRP1 is required for an efficient cell surface exposure of the insulin receptor, we tested if adipose insulin sensitivity is impaired in Arfp1AT–/– mice. For this purpose, 10-week-old animals were acutely injected with insulin or vehicle (NaCl) and sacrificed 20 min later in order to assess the insulin response of white and brown adipose depots (WAT, BAT). Arfp1AT–/– mice revealed a markedly blunted insulin-stimulated AKT phosphorylation in both fat depots, when compared to control littermates (Figure 5A–C). Furthermore, elevated triglyceride, NEFA, and glycerol levels detected in plasma of 10-week-old Arfp1AT–/– mice pointed towards an impaired insulin-mediated suppression of adipocyte lipolysis (Figure 5D–F). To evaluate whether impaired adipose insulin signaling of Arfp1AT–/– mice caused the alterations in plasma lipid levels, we analyzed ex vivo lipolytic activity of gonWAT explants generated from 7-week-old Arfp1AT–/– mice pointed towards an impaired insulin-mediated suppression of adipocyte lipolysis (Figure 5G). Treatment with the β3-adrenergic receptor antagonist isoproterenol markedly stimulated NEFA mobilization from explants of both genotypes, however to a lesser extent from those of control mice indicating an elevation of basal lipolysis (Figure 5G). Treatment with the β3-adrenergic receptor antagonist isoproterenol markedly stimulated NEFA mobilization from explants of both genotypes, however to a lesser extent from those of control mice indicating an elevation of basal lipolysis (Figure 5G).

The western blots showed a tendency towards higher ATGL levels in gonWAT samples of Arfp1AT–/– in comparison to control mice (p = 0.098), which is in agreement with the increased basal lipolytic rate observed in these mice (Figures S8A and B). In addition, presumably due to an impaired insulin receptor signaling, Arfp1AT–/– mice revealed decreased phospho-FOXO1 levels (Figures S8C and D). In line with that, and with reference to the lipodystrophic phenotype of the conditional fat-specific Arfp1 knockout mouse [10], expansion of adipose tissue was found to be impaired in Arfp1AT–/– mice.
Figure 3: Suppression of Arfp1 results in impaired endosomal-mediated secretion at the level of exocytosis. (A) Distribution of the transferrin receptor (TfR, green) and its ligand transferrin (Tf, red) was analyzed in HeLa cells transfected with nt (non-targeting, black bars)- or Arfp1-siRNA (white bars) following 30 min of Alexa568-Tf incubation at 37 °C. Cells were co-stained for ARFRP1 (pink). Scale bar: 20 μm. White arrowheads indicate Tf-TfR signals in the tip region. (B) Tf-TfR distribution was classified and counted. Cells per classification were expressed as percentage of total number of cells. (C, D) Tf-TfR exocytosis was assessed in transfected HeLa cells. At indicated time points, cells were fixed and stained for ARFRP1 (pink). Alexa568-Tf intensity was quantified per cell and expressed as fold of control cells (0 min) set to 1. The relative amount of released Tf was calculated as a measure of Tf-TfR exocytosis (D). (E, F) Tf surface and uptake was analyzed in transfected HeLa cells. Before and after temperature switch, cells were fixed and co-stained with anti-TfR (green) and anti-ARFRP1 (pink). Alexa568-Tf intensity was quantified per cell and expressed as fold of control cells (4 °C) set to 1. The ratio between Tf uptake to Tf surface was calculated as a measure of Tf-TfR endocytosis (F). For all stainings DAPI (blue) was used to visualize nuclei. Scale bar: 20 μm. All quantifications represent mean ± SEM from 3 to 4 independent experiments each with 10–25 images analyzed per condition, n.s. - not significant, *P < 0.05 by unpaired Student’s t-test (B, D, F) or by paired t-test (D, F).
As a consequence, body weight gain of these animals was moderately reduced (Figure 5I), whereas development of lean mass was indistinguishable between the genotypes (Figure S6). Histological examination of gonWAT and scWAT sections from 7- and 10-week-old mice (Figure 5L, M) and subsequent quantification of gonadal adipocyte area confirmed that adipocyte enlargement by hypertrophic growth was blunted in Arfrp1iAT/C0/C0 mice (Figure 5K). From these data, we conclude that reduced cell surface localization of the insulin receptor leads to diminished adipose insulin sensitivity, which, in turn, promotes adipocyte lipolytic activity resulting in attenuation of adipose tissue growth in Arfrp1iAT/C0/C0 mice. We furthermore conclude that adiponectin release and insulin receptor recycling utilize the same post-Golgi trafficking pathways in adipocytes.

### 3.6. Adipocyte-specific disruption of Arfp1 impairs liver insulin signaling and hepatic control of glucose homeostasis

Since we demonstrated that ARFRP1 is necessary for an adequate secretion of adiponectin, which has insulin-sensitizing properties on peripheral tissues such as liver and skeletal muscle [23,24], we assessed insulin sensitivity of these particular tissues. We found that insulin-stimulated Akt phosphorylation was dramatically reduced in livers of Arfp1iAT/−/− mice, whereas insulin sensitivity of skeletal muscle was unaffected (Figure 6A–C). Adiponectin has been described to enhance hepatic insulin response by an increase in phosphorylation and activity of AMPK [24]; however, the level of phosphorylated AMPK assessed in liver lysates was not different between the genotypes (Figure S7). Beside AMPK-related effects, adiponectin was shown to sensitize the liver towards the action of insulin via a selective upregulation of hepatic IRS-2 protein expression [25]. Indeed, we found that IRS-2 protein was significantly diminished in liver lysates of Arfp1iAT/−/− compared to control mice (Figure 6D,E), supporting our assumption that deficiency of circulating adiponectin deteriorates hepatic insulin sensitivity of Arfp1iAT/−/− animals via a selective reduction in hepatic IRS-2 content. In contrast, protein expression of IRS-1 and insulin receptor showed no alterations between the genotypes (Figure 6D,E). Since insulin signaling in the liver largely controls hepatic glucose production, we performed pyruvate tolerance tests following 18 h of fasting. As shown in Figure 6F,G, Arfp1iAT/−/− animals displayed considerably higher blood glucose excursions than control littermates, indicating that gluconeogenesis...
Figure 5: Disruption of Arfrp1 in adipocytes affects insulin sensitivity, lipolytic rate and expansion of adipose tissue. (A, B) In vivo AKT phosphorylation in subcutaneous white (A) and brown (B) adipose tissue (WAT, BAT) of 10-week-old Arfrp1flox/flox (−/−; white symbols) and Arfrp1flox/+ mice (f/f; black symbols) 20 min after injection of insulin (Ins, 1 IU/kg body weight, n = 3–5 mice per genotype) or vehicle (NaCl, n = 2 mice per genotype). (C) Band intensities (insulin treatment) were quantified by densitometric analysis, and the ratio of phosphorylated (pAKT) to total AKT (tAKT) levels was expressed as fold of control set to 1. (D–F) Levels of triglycerides (D), non-esterified fatty acids (NEFA) (E) and glycerol (F) were measured in plasma of 10-week-old mice (n = 10 mice per genotype). (G) Ex vivo lipolysis was assessed under indicated conditions in gonadal WAT explants generated from 7-week-old animals by determining NEFA concentration in the supernatant. Data were normalized to corresponding protein content and expressed as fold of untreated controls set to 1 (n = 7–8 mice per genotype). (I, J) Gain of body weight (I) and fat mass (J) of Arfrp1flox/flox and control mice (n = 14–17 mice per genotype). The arrows indicate start of tamoxifen treatment. (K) Quantification of gonadal adipocyte area from 7- and 10-week-old mice was performed by an automated image analysis software (WimAdipose, Wimasis GmbH) (n = 3–5 mice per genotype and age, each with at least 3 independent histological sections). (L, M) Representative H&E stainings of gonadal and subcutaneous white adipose tissue sections from 7- (L) and 10- (M) week-old animals. Arrowheads indicate areas with smaller adipocytes. All values represent mean ± SEM, n.s. = not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 by unpaired Student’s t-test (C–G, K) or by two-way ANOVA with Bonferroni’s post-test for multiple comparison (I, J).
was increased in these mice. Accordingly, mRNA expression of $G6pc$ was significantly higher in livers of 6 h fasted $Arfrp1^{AT−/−}$ than in control mice further arguing for an elevation in hepatic glucose production via gluconeogenesis (Figure 6H). As a consequence, blood glucose concentration was significantly higher in mice lacking $Arfrp1$ in adipose tissue after 6 h of fasting, an effect which became even more pronounced after 16 h of food deprivation (Figure 6I). However, it cannot be excluded that the elevated blood glucose concentration in $Arfrp1^{AT−/−}$ mice is also a consequence of an impaired glucose uptake by skeletal muscle and adipose tissue. Taken together, the present data strongly suggest that the impaired release of adiponectin from $Arfrp1$-deficient adipose tissue led to perturbations in the adipose hormone axis. This was characterized by an impaired hepatic insulin sensitivity associated to decreased IRS-2 protein levels, which, in turn, resulted in elevated hepatic gluconeogenesis and increased blood glucose levels in $Arfrp1^{AT−/−}$ mice under fasting conditions.

4. DISCUSSION

In the current study, we have identified adiponectin secretion and insulin receptor surface targeting to largely rely on the action of functional ARFRP1 at the trans-Golgi in mature adipocytes. Both cargo molecules utilize trans-endosomal routes for cell surface delivery resulting in either secretion (adiponectin) or plasma membrane exposure (insulin receptor). Mice with an inducible deletion of $Arfrp1$ in adipocytes reveal significantly less adiponectin release and insulin receptor surface localization causing hepatic insulin resistance and impaired glucose homeostasis as well as reduced adipose insulin signaling promoting lipolytic activity and inhibiting adipose tissue expansion. Adiponectin, a classically secreted adipokine depending on proper ER/ Golgi function, has been reported to exit the cell via an ER-Golgi network where it mediates the sorting of selected cargo-containing transport vesicles (trimer, hexamer, high-molecular weight adiponectin) involves a complex set of regulatory steps in the ER and Golgi apparatus[17,27]. Once adiponectin reaches the TGN, a pool of adiponectin molecules is packaged into GGA1 (Golgi-localized, ARF-binding protein 1)-coated vesicles (GGA1) for further delivery to endosomes[31]. In agreement with that, cellular adiponectin has been shown to partially co-localize with Rab11, a marker of

Figure 6: Loss of adipocyte-specific $Arfrp1$ results in deteriorated liver insulin sensitivity, increased hepatic glucose production, and elevated fasting blood glucose levels. (A, B) Western blot analysis of phosphorylated (pAKT) and total AKT (tAKT) levels in liver (A) and quadriceps (B) of 10-week-old $Arfrp1^{trans}$ mice (white symbols) and control mice ($Arfrp1^{fl/fl}$, black symbols) following injection with insulin (Ins, 1 IU/kg body weight, $n = 3−5$ mice per genotype) or vehicle (NaCl, $n = 2$ mice per genotype). (C) Band intensities (insulin treatment) were quantified by densitometric analysis, and the ratio of pAKT to tAKT was expressed as fold of control set to 1. (D, E) Expression of indicated proteins was determined in liver lysates of 10-week-old animals via immunoblotting (D). Signal intensities were quantified by densitometric analysis and expressed as fold of control set to 1 ($n = 3−5$ mice per genotype) (E). (F) Ten-week-old mice were subjected to pyruvate tolerance tests (PTT), and blood glucose concentration was determined at indicated time points (n = 17−20 animals per genotype). (G) The area under the curve (AUC) is depicted for blood glucose. (H) Transcript levels of gluconeogenic genes ($G6pc$, Pck1) were determined by qRT-PCR in livers of 10-week-old animals ($n = 4−18$ mice per genotype) after 6 h of fasting. (I) Blood glucose concentration was measured in 10-week-old mice at indicated time points (n = 10−17 mice per genotype). All data represent mean ± SEM, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 by unpaired Student’s t-test (C, E, G-I) or by two-way ANOVA with Bonferroni’s post-test for multiple comparison (F).
recycling endosomes as well as with Rab5 and EEA1 (early endosome antigen 1) representing early endosomes [18,32].

Based on assays employing the transferrin receptor (TfR) and its ligand transferrin (Tf), which have been widely used to trace endocytic and recycling processes, we demonstrated in the current study ARFRP1 to be critically involved in the process of endosomal-mediated exocytosis (Figure 3C,D). Consistently, adipocyte-specific ablation of Arfrp1 in mice (Figure 2A,B) or 3T3-L1 cells (Figure 2C,D) promoted an endosomal enrichment of adiponectin, showing increased co-localization with Rab11-positive recycling endosomes when Arfrp1 was depleted in 3T3-L1 adipocytes (Figure 2E,F). In accordance with accumulating adiponectin in fat cells, the endosomal transit of the Tf–TfR complex was impaired in Arfrp1-deficient HeLa cells as indicated by accumulation of Tf–TfR at the cell periphery in these cells (Figure 3A,B). From these data, we conclude that the loss of Arfrp1, in adipocytes selectively impairs secretion of adiponectin via trans-endosomal routes.

Further supportive of this assumption is the finding that a fraction of intracellular adiponectin overlaps with TfR-positive membranes in 3T3-L1 adipocytes, implying that adiponectin release occurs via the TR-positive endosomal compartment [26]. Beside this, adiponectin was also described to exit the cell constitutively [18]. However, this route of secretion is principally intact in Arfrp1-depleted cells (Figure S5) and might explain the residual adiponectin that could be detected in plasma and fat explant supernatant of Arfrp1MT/C0 mice.

Notably, both circulating adiponectin and adipisin were equivalently diminished by about 50% in Arfrp1MT/C0 mice consistent with several studies reporting overlapping trafficking routes for their secretion from 3T3-L1 cells which was strikingly inhibited upon ablation of TfR-positive endosomes [18,26,33]. In agreement with an unaffected leptin secretion (Figure 1A,E), post-Golgi traffic of leptin was shown to occur independently of intact endosomal compartments as their inactivation did not compromise leptin release from 3T3-L1 or primary rat adipocytes [18]. The fact that deletion of Arfrp1 reduces the plasma levels of adiponectin and adipisin could indicate that the observed phenotype is the consequence of both adipokines. However, the Spiegelman group recently reassessed the metabolic role of adipin [34] showing that adipin has an impact on sustaining insulin production and/or secretion particularly under conditions of diet-induced obesity. As mice in the present study received a standard diet and additionally showed no obvious phenotype concerning plasma insulin levels, further investigations focused on adiponectin.

Interestingly, the previously generated conditional fat-specific Arfrp1 knockout mouse did not exhibit reduced plasma adiponectin levels, as observed in the current study. However, conditional knockout mice failed to develop functional WAT and only displayed residual BAT, still expressing a considerable amount of Arfrp1, which might represent the exclusive adiponectin source in these mice [10]. Different mouse models of genetic adiponectin ablation demonstrated a selective hepatic insulin resistance without any impairments of insulin sensitivity in skeletal muscle, even under standard diet-fed conditions [25,35,36].

In this context, hypoadiponectinemia was shown to reduce hepatic IRS-2 expression [20,31]. In particular, adiponectin transiently increases macrophage-derived plasma IL-6 levels, which, in turn, activate hepatic STAT3 to induce hepatic IRS-2 expression and enhance insulin sensitivity in the liver [20]. Intriguingly, IRS-2 largely determines insulin signaling in the liver during fasting, thereby substantially controlling hepatic glucose production via gluconeogenesis [21].

These findings conclusively connect the plasma adiponectin decline to the impaired hepatic control of glucose homeostasis in these animals. Following defective endosomal-mediated exocytosis, cell surface exposure of the TR was substantially diminished in Arfrp1-depleted HeLa cells (Figure 3E,F). Similarly, the insulin receptor was less abundant at the plasma membrane in primary adipocytes (Figure 4A,B) and 3T3-L1 cells (Figure 4E,F) deficient of Arfrp1. After binding of insulin to its receptor and internalization, receptor-ligand complexes are directed for either lysosomal degradation, a way of downregulating signal reception, or receptors are sorted for reinsertion into the plasma membrane [37,38]. In this context, it was demonstrated that prevention of insulin receptor recycling resulted in an accelerated shunting to a degradative pathway, thereby reducing the amount of total cellular receptors [21]. Indeed, 10-week-old Arfrp1MT/C0 mice revealed substantially diminished protein levels of the mature receptor in gonadotropes, suggesting that impaired re-exposure at the cell surface, as assessed by lower plasma membrane abundance in adipocytes from 7-week-old Arfrp1MT/C0 mice, was accompanied by enhanced receptor degradation (Figure 4C,D).

As expected, the insulin receptor surface deficiency resulted in reduced adipose insulin sensitivity (Figure 5A–C). By promoting triglyceride synthesis and inhibiting lipolysis, insulin signaling enhances overall lipid storage in adipocytes. Consistently, adipose tissue explants from Arfrp1MT/C0 mice exhibited an increased basal lipolytic rate as well as impaired insulin-mediated suppression of isoproterenol-stimulated lipolysis (Figure 5G). Insufficiently repressed lipolysis consequently associated with an impaired hypertrophic growth of adipose tissue depots in Arfrp1MT/C0 mice (Figure 5J). Collectively, these findings demonstrate the requirement of ARFRP1 for mature adipocytes to maintain proper insulin action, thereby determining adipose tissue mass. The importance of insulin in controlling lipolysis and fat mass is further emphasized by mouse models with alterations in the insulin signaling pathway of adipocytes. For example, mice with a tamoxifen-inducible knockout of the insulin receptor in mature adipocytes reveal a substantial decrease of adipose tissue mass due to increased lipolysis and adipocyte apoptosis resembling most of the features of the Arfrp1MT/C0 phenotype [39]. Furthermore, the sorting receptor SORLA was recently identified to interact with the insulin receptor in adipocytes facilitating its redirection to the cell surface and enhancing insulin signal reception. Accordingly, mice overexpressing SORLA specifically in adipocytes revealed higher insulin receptor surface levels and decreased lipolysis, which promotes the acquisition of fat tissue and exacerbates diet-induced obesity, while SORLA knockout mice showed excessive lipolysis and a reduction in WAT mass [40].

Notably, numerous genetic variants encoding proteins involved in vesicular transportation (e.g. ARL15, Hip1, Rab3gap1) have been identified by genome-wide association studies (GWAS) as being associated with type 2 diabetes-related traits in humans [41]. These findings further highlight the relevance of functional intracellular vesicle trafficking in preserving metabolic health.

5. CONCLUSIONS

Collectively, the present study suggests that adiponectin secretion and insulin receptor surface targeting share the same post-Golgi trafficking pathways which are regulated by the ARFRP1-based sorting machinery. In this regard, our results underline the pivotal role of ARFRP1 for mature adipocytes in maintaining adipose tissue mass and glucose homeostasis.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2017.11.011.

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