Research Paper

Weight Loss Upregulates the Small GTPase DIRAS3 in Human White Adipose Progenitor Cells, Which Negatively Regulates Adipogenesis and Activates Autophagy via Akt–mTOR Inhibition

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ABSTRACT

Long-term weight-loss (WL) interventions reduce insulin serum levels, protect from obesity, and postpone age-associated diseases. The impact of long-term WL on adipose-derived stromal/progenitor cells (ASCs) is unknown. We identified DIRAS3 and IGF-1 as long-term WL target genes up-regulated in ASCs in subcutaneous white adipose tissue of formerly obese donors (WLDs). We show that DIRAS3 negatively regulates Akt, mTOR and ERK1/2 signaling in ASCs undergoing adipogenesis and acts as a negative regulator of this pathway and an activator of autophagy. Studying the IGF-1–DIRAS3 interaction in ASCs of WLDs, we demonstrate that IGF-1, although strongly up-regulated in these cells, hardly activates Akt, while ERK1/2 and S6K1 phosphorylation is activated by IGF-1. Overexpression of DIRAS3 in WLD ASCs completely inhibits Akt phosphorylation also in the presence of IGF-1. Phosphorylation of ERK1/2 and S6K1 is lesser reduced under these conditions. In conclusion, our key findings are that DIRAS3 down-regulates Akt–mTOR signaling in ASCs of WLDs. Moreover, DIRAS3 inhibits adipogenesis and activates autophagy in these cells.

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1. Introduction

Long-term weight-loss (WL) interventions, such as prolonged hypocaloric diets and bariatric surgeries, lead to reduced insulin levels, improvement in insulin sensitivity and glycemic homeostasis in formerly obese people and improve glycemic control in individuals with diabetes mellitus type 2 (T2DM) (Klein et al., 2004; Dixon et al., 2012). Although the underlying mechanisms are not precisely understood, one common key effect of these interventions is a long-term caloric restriction (CR) (Klein et al., 2004; Sjöström et al., 2004; Bradley et al., 2012; Knop and Taylor, 2013). Long-term CR, also referred to as dietary restriction (DR), defined as lessening caloric intake (typically by about 30% in rodents and monkeys) without malnutrition is the most robust intervention to extend health and maximum lifespan in most, but not all, laboratory animal models (Speakman and Mitchell, 2011; de Cabo et al., 2014). It is widely accepted that CR protects cells against oxidative damage (López-Lluch et al., 2008) and induces DNA-repair (López-Otín et al., 2013) and recycling processes such as autophagy (de Cabo et al.,
2014). The underlying mechanisms are however not precisely understood. Increasing evidence suggests that reduced growth factor- and nutrient-responsive protein kinase signaling mediate beneficial effects of CR. Conserved CR-responses are reduced growth hormone (GH)/insulin-like growth factor-1 (IGF-1) and insulin signaling (Bartke et al., 2013; Kenyon, 2010). In mammals, GH produced by the pituitary gland induces production and secretion of IGF-1 in the liver, which acts as endocrine regulator. IGF-1 is also produced in peripheral organs by GH-dependent and -independent pathways, which acts locally in paracrine or autocrine fashion (Sonntag et al., 2012; Bartke et al., 2013). The impact of CR on IGF-1 signaling in the periphery is little understood. Another conserved CR-response is reduced activity of the nutrient-responsive protein kinase, mechanistic target of rapamycin (mTOR), associated with lifespan extension in invertebrates and mice (Kapahi et al., 2004; Selman et al., 2009). mTOR forms a network with insulin/IGF-1 signaling, regulating a wide range of cellular processes, such as autophagy, growth, differentiation and metabolism, which are thought to mediate effects of CR (Laplante and Sabatini, 2012). The mechanisms on how CR employs the insulin/IGF-1–mTOR signaling network to influence cellular downstream pathways are the current focus of obesity and aging research.

Adipose tissue is a main organ implicated in regulation of healthspan induced by reduced insulin/IGF-1–mTOR signaling (Broughton and Partridge, 2009). Decreased insulin sensitivity in subcutaneous white adipose tissue (sWAT) due to an age-related deterioration of sWAT is a hallmark of aging (Borkan et al., 1983). Long-term CR leads to reduced adipocyte size and remodeling of body fat composition away from visceral (v) WAT to sWAT (Huffman and Barzilai, 2010; Spekman and Mitchell, 2011). Since sWAT has rather beneficial and vWAT detrimental effects in aging and obesity this contributes to extension of healthspan. While sWAT adipocytes seem to be particularly beneficial for insulin action due to their crucial role in maintaining whole body glucose homeostasis and lipid metabolism, increasing evidence suggests that health benefits of CR exceed those directly associated with weight-loss. Adipocytes arise from adipocyte-derived stromal/progenitor cells (ASCs), which constitute a large pool of precursors, crucial for adipose tissue renewal, homeostasis, expansion and hence function (Berry et al., 2013; Zwierzina et al., 2015). Upon stimulation by insulin, glucocorticoids, cAMP inducers, and additional serum components ASCs enter a differentiation program, referred to as adipogenesis, to acquire their specific functions as adipocytes (Rosen and MacDougald, 2006). According to the current model adipogenesis involves growth arrest, early and terminal differentiation, including morphological changes, lipid accumulation and the expression of fat cell specific genes, such as fatty acid binding protein-4 (FABP4), perilipin and adipokines. The stages of adipogenesis are orchestrated by a transcriptional cascade involving the adipogenic key factor nuclear receptor peroxisome proliferator-activated receptor-γ2 (PPARγ2) and members of the CCAAT/enhancer-binding protein (C/EBP) family.

The impact of WL on ASCs is unknown. By comparing ASCs from abdominal sWAT of normal weight (NWD), obese (OD) and long-term weight-losing formerly obese donors (WLDs) we showed that long-term WL amongst others reduced the adipogenic activity in these cells (Mitterberger et al., 2014b). To better understand the impact of long-term WL on human ASCs, we compared gene expression in a well characterized ASC population (Mitterberger et al., 2012; Zwierzina et al., 2015) isolated from sWAT of age-matched NWDs, ODs, and WLDs using microarray gene expression analysis. Intriguingly, two strongly induced WL target genes were insulin-like growth factor 1 (IGF-1), the activator of signaling from the IGF-1–receptor, and GTP-binding RAS-like 3 (DIRAS3) (Yu et al., 1999), an imprinted tumor suppressor gene. DIRAS3 encodes a small GTPase which was shown to inhibit signaling through phosphatidylinositol-3-kinase (PI3K) and Ras/Ras-related protein kinase (MAPK) in tumor cells (Luo et al., 2003) and induces a dwarf phenotype in transgenic mice (Xu et al., 2000).

2. Material and methods

2.1. Donors

Human sWAT samples were taken from persons undergoing routine abdominoplasty at the Institute for Plastic and Reconstructive Surgery (Medical University Innsbruck) (Mitterberger et al., 2010, 2011, 2012, 2014a, 2014b). The patients gave their informed written consent and had been approved by the ethical committee of Innsbruck Medical University, Austria, according to the Declaration of Helsinki. All sWAT samples were obtained from the lower abdomen. Obesity and normal weight were defined according to the World Health Organization criteria on the basis of the body mass index (BMI = weight [kg]/height [m²]). Female donors were divided into three groups according to their BMI, obese (OD) (BMI ≥ 30 kg/m²), normal weight (NWD) (BMI 19–25 kg/m²), and long-term weight losing initially obese (WLD) (former BMI ≥ 30 kg/m² and current BMI ≤ 25 kg/m²). None of the women had diabetes, liver, renal, or other severe metabolic diseases. Long-term WL was achieved either by hypocaloric diet or laparoscopic adjustable gastric banding (LAGB). The detailed WL regimens are described in Mitterberger et al. (2010). The women were age matched. The clinical and anthropometric parameters are indicated in Table S1.

2.2. Mouse xenograft studies

Mice were treated in accordance with the guidelines of the “European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes” and the Austrian law. Animal experiments were approved by the ethics committee of the Austrian Federal Ministry of Science and Research (Application No. Zl. 188809/13). Further details are explained in the supplementary experimental procedure.

2.3. Isolation of ASC from human subcutaneous adipose tissue

ASCs were isolated as described (Mitterberger et al., 2012).

2.4. Cell culture

ASCs were cultivated as described (Mitterberger et al., 2012).

2.5. Adipogenic differentiation

Adipogenic differentiation was conducted as described (Mitterberger et al., 2012).

2.6. Retroviral gene expression system

See supplementary experimental procedures.

2.7. Laser scanning confocal indirect immunofluorescence microscopy (IF-CLSM)

IF-CLSM was performed as described (Mitterberger et al., 2012).

2.8. Affymetrix microarray gene expression analysis

See supplementary experimental procedures.

2.9. Quantitative RT-PCR analysis

Expression analysis with q-RT-PCR was performed as described (Mitterberger et al., 2012). β-actin was used for normalization. Primer sequences are listed in Table S2.
2.10. Western blot analysis

Western blot analysis was performed as described (Mitterberger et al., 2012). Antibodies used in the study are listed in the supplementary experimental procedures.

2.11. Flow cytometry analysis

Cells were incubated with Lyso-tracker red (Life Technologies) and analyzed by using FACS Canto (BD Biosciences). Data was analyzed employing FlowJo software.

2.12. Multispectral imaging flow cytometry

See supplementary experimental procedures.

2.13. Statistical analysis

Statistical analysis was performed in GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA). The significance of difference between means was assessed by Student’s t test or analysis of variance (ANOVA). Error bars are represented as the mean ± SEM.

3. Results

3.1. DIRAS3 expression is up-regulated upon long-term WL in human ASCs

DIRAS3 was one of the WL target genes emerged from a global gene expression analysis performed on human cs (cell surface)-DLK1(PREF1)/-cs-CD34+/CD90+/CD105+/cs-SMA+/CD45-/CD31-ASCs freshly isolated from sWAT (Mitterberger et al., 2012). Cells from age and sex matched WLDS (hypocaloric diets and laparoscopic adjustable gastric banding (LAGB)), NWDS and ODs (Table 1A) were subjected to whole genome microarray gene expression analysis (Affymetrix Chip U133+2.0). Results revealed a 11.88-fold higher expression of DIRAS3 in ASCs of WLDS relative to NWDS and a 2.20-fold higher expression of DIRAS3 in ASCs from WLDS relative to ODs (Table 1B). Quantitative real time PCR (q-RT-PCR) confirmed an up-regulation of DIRAS3 expression in early passage of ASCs of the WLDS relative to NWDS and ODs (Fig. 1A) and we observed comparatively higher levels of DIRAS3 protein in ASCs freshly isolated from age-matched NWDS, ODs, and WLDs. Note, IGF-1 and DIRAS3 are two selected CR target genes out of a number of genes, which emerged from the mRNA screen (Mitterberger et al., unpublished results). Thresholds for identification of CR genes: Downregulated through CR (+/vs. NW and OW), 50% cut-off. Upregulated through CR (−/vs. NW and OW), 2-fold cut-off. For detailed technical information, see supplementary experimental procedures.

Table 1

(A) Characteristics of the 2 NWDS, 3 ODs and 3 WLDs (2 hypocaloric diets, 1 LAGB) employed in the global gene expression analysis. (B) IGF-1 and DIRAS3 mRNA expression based on DNA microarray expression profiling in ASCs freshly isolated from age-matched NWDS, ODs, and WLDs. Note, IGF-1 and DIRAS3 are two selected CR target genes out of a number of genes, which emerged from the mRNA screen (Mitterberger et al., unpublished results). Thresholds for identification of CR genes: Downregulated through CR (+ vs. NW and OW), 50% cut-off. Upregulated through CR (− vs. NW and OW), 2-fold cut-off. For detailed technical information, see supplementary experimental procedures.

| A | Age (years) | Procedure | BMI [kg/m²] | Delta BMI [kg/m²] | Duration CR (years) |
|---|-------------|------------|-------------|-------------------|---------------------|
| NWD | 31 ± 11 | | 22 ± 3 | 14 ± 3 | 4 ± 2 |
| WLD | 36 ± 9 | 1 × LAGB, 2 × diet | 22 ± 2 | 32 ± 1 | |
| OD | 46 ± 2 | | |

| B | Gene | IGF-1 (fold increase) | DIRAS3 (fold increase) |
|---|------|-----------------------|------------------------|
| WLD/NWD | 23.59 | | 11.8 |
| WLD/OD | 4.20 | | 2.20 |
| OD/NWD | 5.62 | | 5.43 |

3.2. DIRAS3 negatively regulates Akt–mTOR pathway in human ASCs

DIRAS3 can down-regulate PI3K–Akt–mTOR signaling in cancer cells by inhibition of PI3K activity (Luo et al., 2003). To assess the role of DIRAS3 in regulation of the Akt–mTOR pathway in human ASCs, we knocked down (KD) DIRAS3 using gene specific shRNAs (Fig. 2A). DIRAS3 KD was confirmed at protein level in the experimental cell line U-2OS overexpressing DIRAS3 (Fig. 2B). All further experiments were conducted in ASCs of WLDs. The effect of DIRAS3 KD was examined in serum starved ASCs and after serum re-stimulation by analyzing the phosphorylation of Akt, S6K1 and 4EBP1. Serum starvation of human ASCs down-modulated Akt and S6K1 phosphorylation (Fig. 2C; see also S. Fig. 1 comparing proliferating cells to 0 h). This down-regulation was not observed upon KD of DIRAS3, Akt–mTOR pathway remained active even in the absence of serum (Fig. 2C, compare lanes 1 and 5). Additionally, we observed a significant higher phosphorylation of Akt, S6K1 and 4EBP1 in DIRAS3 KD ASCs after serum stimulation (Fig. 2C–F). To exclude off-target effects we specifically KD DIRAS3 using a different shRNA sequence and observed similar effects on Akt–mTOR pathway activity (S. Fig. 2A–D). Similar effects were observed using ASCs from different donors. To corroborate our results, we studied the regulation of Akt–mTOR pathway in ASCs infected with lentiviruses overexpressing DIRAS3 (Fig. 2G and H). As expected, Akt–mTOR pathway down-modulation was observed at early time points upon serum re-stimulation in DIRAS3 over-expressing ASCs as shown by reduced phosphorylation of Akt, S6K1 and 4EBP1 (Fig. 2I–L). We conclude that DIRAS3 negatively regulates Akt–mTOR signaling in human ASCs.

3.3. DIRAS3 negatively regulates adipogenesis

Activated insulin/IGF-1 (Boucher et al., 2010) and mTOR signaling (Laplante and Sabatini, 2012) stimulate adipogenesis. Since DIRAS3 slows Akt–mTOR signaling and long-term WL reduces adipogenic differentiation capacity of ASCs in formerly obese humans (Mitterberger et al., 2014b), we investigated whether DIRAS3 impinges on adipogenesis in ASCs. Induction of adipogenic differentiation by a hormone cocktail led to a morphological transformation of ASCs to rounded cells during first 72 h after induction, which is a hallmark of adipogenesis (S. Fig. 3). Differentiation was confirmed by staining fat droplets within the cells by Oil-Red-O (S. Fig. 3). Interestingly DIRAS3 mRNA expression was significantly up-regulated at early stages of adipogenesis, 6 h post-induction, while peak of expression was observed after 24 h followed by a gradual decrease (Fig. 3A). Up-regulation of DIRAS3 expression was dependent on the adipogenesis program, as only the complete adipogenic cocktail induced DIRAS3 expression (S. Fig. 4A). The other two members of DIRAS family, DIRAS1 and DIRAS2, were not markedly up-regulated during adipogenesis (S. Fig. 4B and C). We monitored the activity of Akt–mTOR pathway in the course of adipogenesis in human ASCs by analyzing phosphorylation of Akt and S6K1 (S. Fig. 1). Induction of
adipogenesis up-regulated Akt and S6K1 phosphorylation in the density arrested cells (Fig. 3B). Phosphorylation gradually decreased to its basal level approximately 6 h post-induction. Next, we tracked the phosphorylation of Akt and S6K1 in the first 72 h after induction of adipogenesis in DIRAS3 KD ASCs and detected a marked up-regulation of Akt and S6K1 phosphorylation upon DIRAS3 KD (Fig. 3B). Additionally, down-regulation of DIRAS3 was associated with stronger differentiation of ASCs as revealed by a significant increase of induction of FABP4, perilipin and adiponectin (Fig. 3C) and increased lipid accumulation at day 9 post-induction (Fig. 3E). A homolog of DIRAS3 was not found in mice (Yu et al., 2006).

To study the impact of DIRAS3 on adipogenesis in vivo, we injected DIRAS3 KD human ASCs and control ASCs committed to adipogenesis into the posterior sWAT of SCID mice. As shown in Figs. 3F and G, differentiating ASCs were predominantly found in peripheral regions of the transplants adjacent to mouse sWAT. DIRAS3 KD ASCs showed increased adipocyte differentiation in mouse sWAT relative to controls (Fig. 3H), suggesting that DIRAS3 negatively regulates adipogenesis in sWAT of mammals. In accordance with the pro-adipogenic effect of the DIRAS3 KD, overexpression of DIRAS3 in ASCs diminished adipogenesis as shown by significant decrease in mRNA level of FABP4, perilipin and adiponectin upon DIRAS3 overexpression (Fig. 3I). In addition, the perilipin protein level and lipid droplets were decreased in DIRAS3 overexpressing cells at day 9 post-adipogenesis induction (Fig. 3J and K). We conclude that DIRAS3 negatively regulates adipogenesis in human ASCs.

3.4. Regulation of adipogenesis by DIRAS3 is mediated by C/EBP-β and PPAR-γ2

To better understand the mechanisms on how DIRAS3 regulates adipogenesis, we analyzed the expression of transcription factors C/EBP-β, C/EBP-α and PPAR-γ2, which are key regulators of adipogenesis (Rosen and MacDougald, 2006). DIRAS3 KD led to a significant up-regulation in the expression of all three genes in ASCs undergoing adipogenic differentiation (Fig. 4A). Translation of the C/EBP-β mRNA gives rise to three different isoforms, C/EBP-β Full-LAP and C/EBP-β LAP, which are activating isoforms and C/EBP-β LIP, which is an inhibitory isoform (Lechner et al., 2013). C/EBP-β induces the expression of PPAR-γ2 and C/EBP-α, which are the two key regulators of terminal adipogenesis (Park et al., 2004). We analyzed C/EBP-β protein levels and observed higher levels of C/EBP-β Full-LAP and -LAP in the first 72 h of induction upon DIRAS3 KD (Fig. 4B, left panel), while no marked difference was detected in C/EBP-β LIP protein level (Fig. 4B, right panel). Differential migration of CEBP-β Full-LAP and -LAP bands in SDS PAGE corresponds to differential phosphorylation. Activated extracellular signal regulated kinase 1/2 (ERK1/2) was shown to phosphorylate T188 residue of C/EBP-β and thus activate the transcription factor (Park et al., 2004). In accordance, Western blot analysis revealed a marked up-regulation of ERK1/2 phosphorylation during the first 72 h of adipogenesis in DIRAS3 KD ASCs (Fig. 4C). The transcription factor Foxo1 negatively controls PPAR-γ2 expression when localized within the nucleus (Nakae et al., 2003). Foxo1 is negatively regulated by Akt, as its phosphorylation by active Akt leads to its exclusion from the nucleus (Brunet et al., 1999). As DIRAS3 KD is associated with an increased phosphorylation of Akt (Figs. 2C and 3B), we analyzed changes in Foxo1 phosphorylation in ASCs. In fact, DIRAS3 KD led to an up-regulation of Foxo1 phosphorylation (Fig. 4D) and IF-CLSM showed reduced nuclear localization of Foxo1 in DIRAS3 KD ASCs relative to controls (Fig. 4E). This suggests that activated Akt induced by nuclear exclusion of Foxo1 contributes to increased PPAR-γ2 expression upon DIRAS3 KD. Inhibition of Akt and ERK by specific chemical inhibitors diminished DIRAS3 KD mediated up-regulation of adipogenesis, underscoring that active Akt and ERK2/1 execute positive regulation of adipogenesis via C/EBP-β and PPAR-γ2 respectively upon DIRAS3 KD (S. Fig. 5A and B).

3.5. DIRAS3 regulates autophagy in human ASCs

Increased autophagy is one conserved CR-response induced by reduced insulin/IGF-1–mTOR signaling (Laplante and Sabatini, 2012) and DIRAS3 was shown to induce autophagy in cancer cells (Lu et al., 2008). To test whether down-regulation of Akt–mTOR signaling by DIRAS3 stimulates autophagy in human ASCs, we initially conducted Western blot experiments in DIRAS3 KD and overexpressing cells to monitor the cleavage of LC3I to LC3II as a marker for increased autophagy (Klionsky et al., 2012). LC3I and LC3II levels were examined in serum
starved ASCs and in the first hour following serum replenishment, DIRAS3 KD significantly reduced LC3I to LC3II conversion in both conditions (Fig. 5A), while over-expression of DIRAS3 significantly enhanced levels of LC3II cleaved form (Fig. 5B), suggesting a role of DIRAS3 in autophagy induction. To prove that autophagic activity includes flux through the entire system, including lysosomes, and the subsequent release of the degradation products, we analyzed autophagy flux using bafilomycin A1, an inhibitor of lysosomal degradation, resulting in accumulation of LC3II. Under these conditions we found an increased accumulation of LC3II in DIRAS3 overexpressing ASCs, while in DIRAS3 KD LC3II accumulation was markedly reduced (Fig. 5C). The cysteine protease ATG4 is necessary for the processing of LC3I to LC3II and DIRAS3 is associated with ATG4 expression in ovarian cancer cells (Lu et al., 2008). In keeping with this a significant decrease in ATG4 protein level was detected upon

![Figure 2](image-url)
Fig. 3. DIRAS3 is a negative regulator of adipogenesis in human ASCs. (A) DIRAS3 mRNA expression normalized to actin was investigated by q-RT-PCR during the course of adipogenesis (n = 6). (B) Akt–mTOR pathway activity pattern was monitored during the first 72 h of adipogenesis upon DIRAS3 KD. Phosphorylation of Akt (S473) and S6K1 (T389) was examined by Western blotting. (C) ASCs infected with either shCntrl or shDIRAS3 expressing lentiviruses were subjected to adipogenesis and mRNA expression of adipocyte marker genes FABP4, Perilipin and Adiponectin normalized to actin were analyzed using q-RT-PCR at the indicated time points. (D) Perilipin protein expression was analyzed by western blotting at day 9 post-adipogenesis induction in shCntrl and shDIRAS3 ASCs. (E) Adipocyte differentiation was estimated using Oil-Red-O staining at day 9 post-induction. (F and G) Representative immunohistochemical staining of xenotransplanted shDIRAS3 SCID mice (F) and shCntrl mice (G) using anti-perilipin antibodies. Region of Interest (ROI) is shown in higher magnification. (H) Margin of the transplant from each mice was imaged at 20× magnification and perilipin positive and negative cells were counted using ImageJ cell counter plugin and shown as percentage positive cells (n = 6). (I–K) ASCs infected with either Mock or DIRAS3 overexpressing lentiviruses were subjected to adipogenesis and mRNA expression of adipocyte marker genes normalized to actin was analyzed using q-RT-PCR at the indicated time points (I). Perilipin protein analysis (J) and Oil-Red-O staining (K) were done at day 9 post-induction of adipogenesis. All error bars represent the means ± SEM. p values * = p < 0.05, ** = p < 0.001 and *** = p < 0.0001. The significance of difference between means was assessed by analysis of variance (ANOVA) (A, C and I) and Student’s t test (H).
DIRAS3 KD (Fig. 5D), underscoring that DIRAS3 stimulates autophagy in ASCs. Moreover, visualization of autophagic punctate employing GFP-LC3 as marker for autophagosome detection, demonstrated that knock-down of DIRAS3 in GFP-LC3 expressing ASCs led to a decreased punctate formation (Fig. 5E). In contrast, DIRAS3 over-expression enhanced accumulation of punctate spots (Fig. 5F). To quantitate cells displaying high autophagic puncta we employed image stream multi-spectral flow cytometry (Klionsky et al., 2012). Human ASCs co-infected with lentiviruses expressing GFP-LC3 were serum-starved (positive control) or not starved (negative control) and monitored (S. Fig. 6I). We defined a cell population having more than 3 puncta as high autophagy cells. A 15-fold increase in cell number displaying high autophagy upon starvation was observed when compared to cells incubated in 10% FCS (non-induced) (Fig. 5G). Next, ASCs were co-infected with lentiviruses expressing GFP-LC3 and DIRAS3 shRNA or control shRNA. DIRAS3 KD led to a significant decrease in the number of cells displaying high autophagy (Fig. 5H and S. Fig. 6J). Another hallmark of autophagy is development of acidic vesicular organelles which can be detected by FACS using lysotracker red staining. Consistent with Western blot data (Fig. 5A–C), we observed significantly lower fluorescence intensity in DIRAS3 KD ASCs (Fig. 5I) and significant higher fluorescence intensity in DIRAS3 OV ASCs (Fig. 5J), indicating that DIRAS3 level is one of the determinants of autophagy. Furthermore, we demonstrate regulation of autophagy related genes upon DIRAS3 KD (S. Fig. 6A–D) and DIRAS3 overexpression (S. Fig. 6E–H). We conclude that DIRAS3 regulates autophagy in human ASCs. Having shown that WLDs display an increased DIRAS3 expression in their ASCs and abdominal sWAT (Fig. 1A, B and Table 1) and that increased DIRAS3 expression is linked to higher autophagy in ASCs (Fig. 5A–J), we investigated LC3I/LC3II protein level in the WAT lysates of the given donor groups. We found a higher LC3II to LC3I ratio in adipose tissue lysates from WLDs relative to NWD and OD, confirming elevated induction of autophagy (Fig. 5K). Together our results highlight a role of DIRAS3 in autophagy induction upon long-term WL in humans ASCs.
Impaired adipogenesis in ATG5 and ATG7 knock-out mice indicates that autophagy is essential for adipogenesis (Baeria et al., 2009; Zhang et al., 2009). IGF-1–mTOR signaling plays an important role in the regulation of both adipogenesis and autophagy; the inhibition of IGF-1–mTOR pathway induces autophagy but reduces adipogenesis (Laplante and Sabatini, 2012; this study). To better understand the interaction between IGF-1–mTOR and autophagy activity in adipogenesis, we monitored the conversion of LC3I to LC3II in human ASC during differentiation. We found a consistent induction level of autophagy in the course of adipogenesis, demonstrating a general role of autophagy in this process (S. Fig. 1). However, upon DIRAS3 KD we detected a decrease in autophagic activity (Fig. 5L) incidental to up-regulated Akt–mTOR signaling (Fig. 3B) and increased adipogenesis (Fig. 3C–E). Thus, although autophagy is essential for adipogenesis in ASCs an activated Akt–mTOR pathway can obviously drive this process at reduced but sufficient autophagic activity.

3.6. Long-term WL induces IGF-1 expression in human ASCs

Another WL target gene which emerged from our microarray analysis was IGF-1, the activating ligand of the IGF-1 receptor (IGF-1-R). IGF-1 showed a 23.59-fold up-regulation in ASCs of WLDs versus NWDs and a 4.20-fold higher expression in ASCs from WLDs relative to ODS (Table 1B). These results were confirmed in early passage ASCs from WLD, ODS and NWD donors by qRT-PCR (Fig. 6A). The further experiments were conducted in ASCs from WLDs. Serum-starvation of proliferating ASCs induced IGF-1 mRNA expression (Fig. 6B), supporting our in vivo observed IGF-1 up-regulation upon WL. Furthermore, we corroborated that ASCs derived from swAT secrete IGF-1 in culture (Fig. 6C). These findings were intriguing, because reduced IGF-1 receptor level was reported in adipose (Breeze et al., 1999; Scantag et al., 1999) and reduced insulin/IGF-1 signaling in invertebrates (Fontana et al., 2008). There is also precedence for IGF-1–mTOR signaling as a conserved CR response (Broughton and Partridge, 2009). Since IGF-1 stimulates differentiation of preadipocytes (Boucher et al., 2010; Poulos et al., 2010), its expression during adipogenesis was investigated in human ASCs. We found a significant up-regulation of IGF-1 between days 1 and 3 post-induction of adipogenesis (Fig. 6D). A sharp decrease in the IGF-1 expression was observed at day 4 after exclusion of IMRX from the medium, suggesting that the adipogenesis program drives the increase in IGF-1 expression (Fig. 6D). Concomitant with increased IGF-1 mRNA level, we found significantly increased IGF-1–mTOR expression upon serum starving ASCs (Fig. 6E) and during adipogenesis (Fig. 6F). The induction of DIRAS3 in adipogenesis followed a similar time course (Fig. 3A). We also found that exogenous addition of IGF-1 leads to a slight induction of DIRAS3 in WLD ASCs (Fig. 6G). This suggests that positive and negative regulators of IGF-1 signaling are induced during adipogenesis and obviously also in normal ASCs. In the course of adipogenesis, in pro-adipogenic medium, both the MAPK (Fig. 4C) and the PI3K–mTOR pathways (Figs. 2C and 3C) are strongly activated and DIRAS3 can inhibit both of them. To better understand the interaction between IGF-1 and DIRAS3 from ASCs to WLDs, we studied the biochemical interaction between the two proteins in regulating Akt, ERK1/2 and mTOR pathway. To do this, we added solely IGF-1 in concentrations at 0.5 × 10⁻⁹ M–2.5 × 10⁻⁹ M, which are in the physiologically range for the stimulation of human preadipocytes (Bäck and Arnlqvist, 2009), to WLD ASCs infected with mock or DIRAS3 overexpression lentiviruses. Although IGF-1 is strongly up-regulated in ASCs from WLD (Table 1B, Fig. 6A), IGF-1 could hardly induce phosphorylation of Akt in mock WLD ASCs (Fig. 6H, left panels). Phosphorylation of S6K1 and ERK1/2 was induced by IGF-1 in these cells (Fig. 6H, right panels). Overexpression of DIRAS3 in WLD ASCs completely inhibited phosphorylation of Akt also in the presence of IGF-1 (Fig. 6H, right panels). Phosphorylation of ERK1/2 was less reduced under these conditions and S6K1 remained also active to some extent.

4. Discussion

Transgenic mice overexpressing the human DIRAS3 gene show decreased body size, reduced development in multiple organs and are proportionally smaller than nontransgenic littermates (Xu et al., 2000). This phenotype is similar to calorically restricted mice, which showed reduced insulin and IGF-1 serum levels (Speakman and Mitchell, 2011). In the present study, we identified DIRAS3 as WL target gene up-regulated in ASCs of formerly obese humans upon long-term WL and demonstrated that DIRAS3 reduces adipogenic differentiation in these cells. Under pro-adipogenic conditions DIRAS3 strongly counteracted activation of Akt and mTOR signaling. Moreover, although IGF-1 expression was up-regulated in the course of adipogenesis DIRAS3 reduced adipogenic differentiation. Thus, DIRAS3 counteracted insulin and IGF-1 activity during adipogenesis. Additionally, DIRAS3 decreased adipocyte differentiation product levels (FABP4, perilipin and adiponectin) in immature adipocytes. These data suggest that DIRAS3 activity down-modulates adipogenesis and anabolic processes in swAT of WLDs. This is in accordance with the previously shown reduced adipogenic capacity of ASCs explanted from swAT of WLDs (long-term restricted by hypocaloric diet or bariatric surgery) (Mitterberger et al., 2014b). Insulin levels in serum of WLDs are significantly lower than those in serum of ODS and NWDs (Mitterberger et al., 2010, 2011). Given the importance of insulin as a potent positive regulator of adipogenesis, it is likely that increased DIRAS3 levels further add to a reduced adipogenic activity in ASCs of swAT of formerly obese donors. Both insulin and IGF-1 can in principle stimulate adipogenesis in adipose progenitor cells, which express the highly homologous insulin receptors (IR) and IGF-1 receptors (IGF-1R) at their surface (Bäck and Arnlqvist, 2009; Boucher et al., 2010). While insulin and IGF-1 bind preferentially to their cognate receptors the two hormones can induce adipogenesis via activation of both the IR and IGF-1R in preadipocytes. To clarify the importance of insulin in obesity, WL and CR it should be noted that insulin serum levels are more dynamically regulated than IGF-1 serum levels in both conditions. Moreover, both IR and IGF-1R are present at adipocytes, with considerably more IR on these cells, leading to a lipodystrophic phenotype in IR knockouts (KOs) in adipose (Blüher et al., 2002), while IGF-1R KO in adipose seem...
to show mild expansion of adipose (Klöting et al., 2008). Thus WL or CR-induced, reduced insulin serum concentrations count also strongly for beneficial metabolic effects at the adipocyte level, such as reduced lipid depots. When the IR (Blüher et al., 2002) or IGF-1R (Klöting et al., 2008) is inactivated in adipose tissue in mice these pathways can however at least in part compensate for each other.

Intriguingly, in the present study, we found that in addition to DIRAS3, IGF-1, the activating ligand of the IGF-1R, was considerably induced in ASCs from WLDs. We showed however that addition of IGF-1 to WLD ASCs hardly stimulated Akt phosphorylation. Moreover, DIRAS3 overexpression completely inhibited Akt phosphorylation also in the presence of high levels of IGF-1. IGF-1 stimulated ERK1/2 phosphorylation in the WLD ASCs and S6K1 was also phosphorylated to some extent. In DIRAS3 overexpressing cells ERK1/2 and S6K1 remained active under these conditions. Our data suggest that DIRAS3 dominantly down-regulates the IGF-1R–Akt–mTOR branch in WLD ASCs. Moreover, it is conceivable that the functional interaction between IGF-1 and DIRAS3 channels the IGF-1 stimulus away from Akt to the ERK1/2 branch. A minimum of IGF-1/IGF-1-R signaling is most likely important to balance down-modulated anabolic processes along with growth, differentiation and survival of ASCs. In fact, it has been shown that extreme low IGF-1 signaling is incompatible with life and supplementation of IGF-1 can ameliorate premature aging in progeria mice with very low levels of IGF-1 (Mariño et al., 2010). Further, in contrast to laboratory animal models, long-term CR does not reduce circulating IGF-1 levels and IGF-1:IGFBP-3 ratio in non-obese humans (Fontana et al., 2008). Similarly, we have shown that total IGF-1 serum levels, free IGF-1 and the molar IGF-1:IGFBP-3 ratio are not significantly lower in WLDs than in ODs (Mitterberger et al., 2011).
The molar ratio between IGF-1:IGFBP-3 differed also not between WLDs and NWDs. IGF-1 serum levels are however lower in WLDs probably due to the former obesity phenotype of these donors. On one hand, excessive growth and differentiation of stem and progenitor cells can be deleterious due to accelerated exhaustion of stem cell niches, a hallmark of aging. On the contrary, excessive autophagy, a diminished growth, differentiation and survival of these cells is a detrimental factor for long-term maintenance of the organism, because of its deleterious effects on regeneration and renewal of tissues (Jones and Rando, 2011). For this reason, the maintenance of a correct balance between the two situations could represent a crucial node in ASC homeostasis. Hereby, we demonstrate a simultaneous up-regulation of IGF-1 and DIRAS3 expression upon WL/CR in ASCs, in combination with reduced insulin serum levels, and almost unaltered serum IGF-1 levels, which supports the hypothesis that WL/CR optimizes the insulin/IGF-1–mTOR signaling system in ASCs of sWAT, to achieve a protecting resting-state. As a consequence, ASCs in sWAT of weight losing or calorically restricted formerly obese individuals will present a higher renewal rate and readiness to grow and differentiate in permissive conditions. This may contribute to maintenance of sWAT in conditions of CR, as shown previously in mouse models (Huffman and Barzilai, 2010), and contribute to postponement of sWAT aging.

Our findings contribute to a better understanding of the role of the insulin/IGF-1 system in CR. In lower eukaryotes disruption of IGF-1–insulin signaling increases lifespan (Kenyon, 2010; Johnson, 2013) and knockouts of genes coding for several components of the GH/IGF-1–mTOR signaling network extend lifespan in rodents (Coschigano et al., 2000; Holzenberger et al., 2003; Taguchi et al., 2007; Selman et al., 2009). Furthermore, CR induced longevity in rodents correlates with decreased IGF-1 serum levels (Sonntag et al., 2012) and a decline in the GH/IGF-1 axis in mice and humans is associated with decreased incidence of cancer (Bartke et al., 2013). In contrast, reduced activity of the IGF-1 pathway, which has diverse physiological functions, leads often to detrimental effects. In fact, reduced IGF-1 serum levels correlate with higher incidence of T2DM, cardiovascular diseases and declining cognitive functions, suggesting that IGF-1 deficiency contributes to the pathology of aging (Broughton and Partridge, 2009; Sonntag et al., 2012). Supporting the notion, circulating IGF-1 levels decrease with age in humans and animals and a reduction in serum IGF-1 levels during aging impairs health span in mice (Gong et al., 2014). The dual, beneficial and deleterious actions of IGF-1 led to the current concept, that IGF-1 signaling is a complex endocrine, paracrine and autocrine network affecting organ development, homeostasis and function throughout life (Broughton and Partridge, 2009; Sonntag et al., 2012). Our study supports the hypothesis that the extent, tissue and cell type specific IGF-1 activity are determinants of the effect on health and lifespan.

IGF-1 is produced by the liver as endocrine hormone and in peripheral tissues in a paracrine/autocrine fashion. Adipose tissue contains more IGF-1 than any other tissues except the liver (Stratikopoulos et al., 2008), it is secreted in WAT in a GH-dependent and -independent manner (Bartke et al., 2013) and by isolated ASCs and adipocytes (Wabitsch et al., 2000; Poulos et al., 2010; this study). Supporting our data that IGF-1 is highly expressed in ASCs of sWAT from WLDs it was demonstrated that IGF-1 levels are up-regulated in peripheral tissues of Ames dwarf mice, which show the typical longevity phenotype of chronically low IGF-1 serum levels (Bartke et al., 2013). In these mice, local production of IGF-1 was demonstrated in the hippocampus (Sun et al., 2005) correlating with neuroprotection (Schrag et al., 2008). Moreover, signaling by insulin-like peptides (ILPs) is beneficial in neurons of flies and worms, despite the paradox that lowered insulin/IGF-1 activity has the potential to both compromise the integrity of the CNS and extend lifespan (Broughton and Partridge, 2009). Furthermore, DR results in reduced expression of Drosophila ILP (DILP) 5 in neurosecretory cells in the brain. Deletion of the genes DILP 2, 3 and 5 in these cells, which extends lifespan, is however accompanied by up-regulation of DILP 6 in the fly fat body (Partridge et al., 2011). Thus, similar to our findings in human ASCs, other studies show increased IGF-1 levels in tissues of mammals and invertebrates in response to either CR/DR or mutations reducing insulin/IGF-1 signaling, which could reflect a compensatory response.

Long-lived mutant flies with reduced DILP signaling show increased loss of germline stem cells (Hsu and Drummond-Barbosa, 2009) while DR induces longevity and enhances germline stem cell maintenance in flies with age (Mair et al., 2010). The different mechanisms by which these apparently related interventions have opposite effects on stem cell maintenance are not precisely understood. Our data suggest that WL/CR optimizes the activity of the IGF-1–mTOR system with beneficial effects on ASCs and sWAT homeostasis.

We demonstrated increased autophagy in sWAT of WLDs and showed that negative regulation of Akt–mTOR signaling by DIRAS3 in human ASCs increases autophagy, a hallmark of CR (de Cabo et al., 2014). This establishes a mechanism how WL/CR employs IGF-1–DIRAS3–mTOR signaling to induce autophagy. Increased autophagy leads to higher turnover of cellular material and recycles misfolded or damaged cellular components. Thus, DIRAS3-induced autophagy in ASCs might facilitate homeostasis of cellular macromolecules, providing new cellular building blocks and energy for renewal and survival of these cells. According to the current model, nutrient overload in obesity stimulates mTOR, thereby attenuating autophagy (Stienstra et al., 2014). Increasing oxidative stress, hypoxia and inflammation induce insulin resistance in adipose tissues of obese people, leading to inhibition of mTOR and consequently stimulation of autophagy. Studies showing increased autophagy in adipose tissue of obese people suggest that stimulatory effects for autophagy prevail over putative inhibitory effects of mTORC1 activation by overeating (Stienstra et al., 2014). Hereby, we have shown that activation of autophagy in sWAT is a response to long-term WL/CR in formerly obese people. Thus, autophagy in fat tissue is induced in both conditions, obesity and WL/CR, and should affect adipose tissue mass and homeostasis; however, most likely by different mechanisms and intensity.

We have shown that long-term WL/CR modulates adipocyte differentiation of ASCs in human sWAT by inducing both positive and negative regulators of the IGF-1 system at early stages of adipogenesis. Importantly, we identified DIRAS3 as novel negative regulator of adipogenesis, which slows down this process by inactivating Akt–mTOR signaling. Congruent with our data, it was shown in other cell systems that mTOR-induced adipogenesis is mediated partly by 4E-BP1 via regulation of the translation of PPARY2 and by S6K1, which regulates the expression of early adipogenic transcription factors including C/EBPβ (Laplante and Sabatini, 2012). Moreover, in line with data showing that activated ERK can phosphorylate and thereby activate C/EBPβ (Park et al., 2004), we showed that DIRAS3 inhibits ERK phosphorylation in ASCs and this correlates with reduced C/EBPβ activation. Pro-adipogenic effects of IGF-1 have been shown in previous studies (Bäck and Arnljotsson, 2009; Boucher et al., 2010). We extended this knowledge showing that IGF-1 can act as positive regulator of adipogenesis in primary human ASCs and simultaneously induces IGF-1R and DIRAS3, indicating an interplay between IGF-1 and DIRAS3 in modulating adipogenesis.

In conclusion, our results suggest that long-term WL-induced up-regulation of DIRAS3 in ASCs in sWAT of formerly obese humans leads to down-regulation of Akt–mTOR signaling in these cells. Moreover, our data suggest that increased DIRAS3 levels in ASCs in sWAT of WLDs contribute to the reduced adipogenic activity found in ASCs explanted from sWAT of these donors. Furthermore, our findings suggest that increased DIRAS3 levels activate autophagy in ASCs. This study is at the interface between aging and obesity research. The translational value of the paper is in its contribution to a better understanding of the molecular mechanisms underlying the effects of long-term WL/CR on adipose tissue in formerly obese people.

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Author contributions

WZ, AE and MCM designed the experiments. AE performed most of the experiments and contributed to writing of the manuscript. MCM, MM, MEZ, SH, HPV, AK, AM and UR performed the experiments. ZL, MM, SK, GP and RCB participated in the design of the study and contributed biospies and/or reagents. WZ conceived the study and wrote the manuscript. All authors read and approved the manuscript.

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