The negative adipogenesis regulator DLK1 is transcriptionally regulated by TIS7 (IFRD1) and translationally by its orthologue SKMc15 (IFRD2)

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Abstract

Delta-like homolog 1 (DLK1), an inhibitor of adipogenesis, controls the cell fate of adipocyte progenitors. Understanding regulatory mechanisms affecting DLK1 levels is therefore of high interest. Here we identify two independent mechanisms, transcriptional and translational, by which TIS7 (IFRD1) and its orthologue SKMc15 (IFRD2) regulate DLK1 levels. Mice deficient in both TIS7 and SKMc15 (dKO) had severely reduced adipose tissue and were resistant to high fat diet-induced obesity. Wnt signaling, a negative regulator of adipocyte differentiation was significantly up regulated in dKO mice. Elevated levels of the Wnt/ β -catenin target protein Dlk-1 inhibited the expression of adipogenesis regulators PPAR γ and C/EBP α , and fatty acid transporter CD36. Although both, TIS7 and SKMc15, contributed to this phenotype, they utilized two different mechanisms. TIS7 acted by controlling Wnt signaling and thereby transcriptional regulation of Dlk-1. On the other hand, here we provide experimental evidence that SKMc15 acts as a general translational inhibitor affecting DLK-1 protein levels. Our study provides data describing novel mechanisms of DLK1 regulation in adipocyte differentiation involving TIS7 and SKMc15.

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45 *Nonstandard abbreviations used:* CD36, cluster of differentiation 36; HFD, high fat diet; IFRD, interferon
46 related developmental regulator; KO, knockout; dKO, double knockout; RD, regular diet; SKMc15, skeletal
47 muscle cDNA library clone15; TIS7, TPA Induced Sequence 7

Introduction

Adipogenesis is a complex process in which multipotent stem cells are converted into preadipocytes before terminal differentiation into adipocytes¹. These mechanisms involve protein factor regulators, epigenetic factors, and miRNAs. TPA Induced Sequence 7 (TIS7) protein has been shown to be involved in the mainly transcriptional regulation of differentiation processes in various cell types, e.g. neurons², enterocytes³, myocytes⁴ and also adipocytes⁵.

Multiple lines of evidence link the regulation of Wnt/ β -catenin signaling to the physiological function of TIS7, known in human as Interferon related developmental regulator 1 (IFRD1)^{6,7}. Our data based on experiments with TIS7 knockout mice show a negative effect of TIS7 on Wnt signaling and a positive on adipocyte differentiation^{6,8}. TIS7 deficiency leads to a significant up regulation of Wnt/ β -catenin transcriptional activity in both primary osteoblasts and in mouse embryonic fibroblasts (MEFs) derived from TIS7 knockout (KO) mice. Recent data show that TIS7 is also involved in the control of adipocytes differentiation in mice and was up regulated in both visceral (vWAT) and subcutaneous (sWAT) white adipose tissues (WAT) of genetically obese ob/ob mice⁵. TIS7 transgenic mice have increased total body adiposity and decreased lean mass compared with wild type (WT) littermates³. On HFD, TIS7 transgenic mice exhibit a more rapid and proportionately greater gain in body weight with persistently elevated total body adiposity. Enhanced triglyceride (TG) absorption in the gut of TIS7 transgenic mice³ indicated that TIS7 expressed in the gut epithelium has direct effects on fat absorption in enterocytes. We have shown that as a result of impaired intestinal lipid absorption TIS7 KO mice displayed lower body adiposity⁸. Compared with WT littermates, TIS7 KO mice do not gain weight when chronically fed an HFD and TIS7 deletion results in delayed lipid absorption and altered intestinal and hepatic lipid trafficking, with reduced intestinal TG, cholesterol, and free fatty acid mucosal levels in the jejunum⁹. TIS7 protein functions as a transcriptional co-regulator¹⁰ due to its interaction with protein complexes containing either histone deacetylases (HDAC)^{4,11,12,13} or protein methyltransferases, in particular PRMT5¹⁴. The analysis of adipocyte differentiation in preadipocytic 3T3-L1 cells suggested an involvement of TIS7 in the regulation of adipogenesis in the Wnt/ β -catenin signaling context⁵.

SKMc15, also known as Interferon related developmental regulator 2 (IFRD2), a second member of TIS7 gene family, is highly conserved in different species ¹⁵. Mouse TIS7 and SKMc15 are highly homologous, with a remarkable identity at both the cDNA and amino acid levels (58% and 88%, respectively). However, there was so far no information about the physiological function and mechanisms of action of SKMc15 and its possible involvement in differentiation of various tissues. Recently, cryo-electronmicroscopy (cryo-EM) analyses of inactive ribosomes identified IFRD2 (SKMc15) as a novel ribosome-binding protein inhibiting translation to regulate gene expression ¹⁶. The physiological function of SKMc15 matching the mechanism based on the above mentioned cryo-EM data was so far not shown and we postulate it could be involved in adipogenesis, since a significant reduction of whole protein synthesis was previously shown as a major regulatory event during early adipogenic differentiation ¹⁷.

Adipogenesis occurs late in embryonic development and in postnatal periods. Adipogenic transcription factors CCAAT/enhancer binding protein α (C/EBP α) and peroxisome proliferator activated receptor γ (PPAR γ) play critical roles in adipogenesis and in the induction of adipocyte markers ¹⁸. PPAR γ is the major downstream target of Delta-like protein 1 (DLK-1). It is inactivated by the induction of the MEK/ERK pathway leading to its phosphorylation and proteolytic degradation ¹⁹. DLK-1, also known as Pref-1 (preadipocyte factor 1) activates the MEK/ERK pathway to inhibit adipocyte differentiation ²⁰. C/EBP α is highly expressed in mature adipocytes and can bind DNA together with PPAR γ to a variety of respective target genes ²¹. Besides that, PPAR γ binding to C/EBP α gene induces its transcription, thereby creating a positive feedback loop ²². Both proteins have synergistic effects on the differentiation of adipocytes that requires a balanced expression of both C/EBP α and PPAR γ .

Wnt/ β -catenin signaling is one of the extracellular signaling pathways specifically affecting adipogenesis ²³, ^{24, 25} by maintaining preadipocytes in an undifferentiated state through inhibition of C/EBP α and PPAR γ ²⁶. PPAR- γ and Wnt/ β -catenin pathways are regarded as master mediators of adipogenesis ²⁷. Wnt signaling is a progenitor fate determinator and negatively regulates preadipocyte proliferation through DLK-1 ²⁸. Mice over-expressing DLK-1 are resistant to HFD-induced obesity, whereas DLK-1 KO mice have accelerated

adiposity²⁹. DLK-1 transgenic mice show reduced expression of genes controlling lipid import (CD36) and synthesis (Srebp1c, Ppar γ)³⁰. DLK-1 expression coincides with altered recruitment of PRMT5 and β -catenin to the DLK-1 promoter³¹. PRMT5 acts as a co-activator of adipogenic gene expression and differentiation³². SRY (sex determining region Y)-box 9 (Sox9), a transcription factor expressed in preadipocytes, is down regulated preceding adipocyte differentiation. DLK-1 prevents down regulation of Sox9 by activating ERK, resulting in inhibition of adipogenesis³³. The PRMT5- and histone-associated protein COPR5, affects PRMT5 functions related to cell differentiation³⁴. Adipogenic conversion is delayed in MEFs derived from COPR5 KO mice and WAT of COPR5 KO mice is reduced when compared to control mice. DLK-1 expression is up regulated in COPR5 KO cells³¹.

Here we show the involvement of TIS7 and SKMc15 in the process of adipocyte differentiation. dKO mice had strongly decreased amounts of the body fat when fed with even regular, chow diet and were resistant against the high fat diet induced obesity. We found two independent molecular mechanisms through which TIS7 and SKMc15 fulfill this function in a synergistic manner. TIS7 regulates the Wnt signaling pathway activity and restricts DLK-1 protein levels, thereby allowing adipocyte differentiation. In contrast, SKMc15 KO did not affect Wnt signaling, but as we show here, cells lacking SKMc15 have significantly up regulated translational activity. This was true also for dKO, but not for the single TIS7 knockout cells. The ablation of both TIS7 and SKMc15 genes significantly affected the expression of genes essential for adipocyte differentiation and function. Since dKO mice render a substantially leaner phenotype on chow diet, even without any challenge by HFD-induction, we propose that TIS7 and SKMc15 represent novel players in the process of physiological adipocyte differentiation.

Results

Mice lacking TIS7 and SKMc15 genes have lower body mass, less fat and are resistant against HFD-induced obesity.

In order to clarify whether both, TIS7 and SKMc15 are involved in the regulation of the adipocyte differentiation and if they act through same or different mechanisms, we have generated mice lacking both

genes by crossing TIS7 with SKMc15 single KO mice. dKO pups were viable, and adult male and female mice were fertile. At birth, body weights of dKO and WT mice were similar. Nevertheless, already during weaning, both the male and female dKO mice failed to gain weight when compared to their WT littermates and this persisted in the following weeks when the mice were fed regular diet (RD; chow diet) containing 11 % kcal of fat. At 10 weeks of age, dKO mice displayed 30 % and later up to 44.9 % lower body weight compared with WT mice (Figure 1a).

Based on Dual Energy X-ray Absorptiometry (DEXA) measurement, 6-month-old WT mice had substantially higher amounts of fat than their dKO littermates (Figure 1b, left panel). The effect of TIS7 and SKmc15 double knockout was even more pronounced when the total fat and lean mass values were normalized to the body weight since the dKO mice were smaller than their WT counterparts. The percentage of fat was in the WT mice 37.7 ± 4 % vs. 6 ± 3 % of the total body mass in dKO animals. Furthermore, the percentage of lean tissue mass in WT animals was lower than in dKO animals (60 ± 4.6 % vs. 92 ± 3.14 %; Figure 1b, right panel). Nevertheless, the dKO mice were not overall smaller, since their body length, including the tail did not significantly differ between WT and dKO mice at different age (n=20; data not shown). Next, we analyzed the contribution of TIS7 and SKMc15 to the whole-body fat content of mice. Three-dimensional reconstruction of images based on micro-computed tomography (micro-CT) of sex/age-matched adult mice disclosed that both TIS7 and SKMc15 single KO mice already had less abdominal fat than WT controls and that the dKO of TIS7 and SKMc15 genes caused the strongest decrease in abdominal fat content and size (Figure 1c). Quantitative analyses of micro-CT measurements showed that TIS7 deficiency caused a substantial lack of the abdominal fat tissues ($P=0.002$ when compared to WT mice, Figure 2a). Whereas TIS7 KO mice had less fat mass but were not significantly lighter than their WT littermates (Figure 2b), SKMc15 KO were lighter ($P=0.007$) and leaner ($P=0.0001$) and dKO mice were both significantly lighter ($P = 0.0001$) and had significantly less abdominal fat ($P=0.006$) than the WT mice (Figures 2a, b).

We conducted an indirect calorimetry trial with sex- and age-matched WT and dKO animals (Supplementary figure 1). We did not identify any significant difference in respiratory exchange ratios ($RER = VCO_2/VO_2$) of

WT and dKO mice (Supplementary figure 1a). However, dKO mice showed substantially reduced body weight, mainly because of lacking fat, despite identical food intake, activity and no major differences in several metabolic parameters. To investigate potential links between TIS7, SKMc15 and obesity, we studied the response of dKO mice to HFD. At 2 months of age, male mice were housed individually and fed with HFD for 21 days. Food intake was measured every second day and body weight was measured every fourth day. Feces were collected every second day to analyze the composition of excreted lipids and blood samples were collected after the third week of HFD feeding to measure the concentrations of hepatic and lipoprotein lipases, respectively. Already within the first week of HFD feeding, WT mice gained more weight than the dKO mice (Figure 2c), although there were no obvious differences in food consumption (Supplementary figure 1b) or in levels of lipolytic enzymes (Supplementary figures 1c, d). These differences in body weight gain continued to increase during the second and third week, at which time the body weight of WT mice increased additionally for 30% ($30.0 \pm 2.3\%$) when compared with the beginning of the HFD feeding period. In contrast, the weight of dKO animals increased only slightly ($7.6 \pm 1.6\%$) (Figure 2c). Both genes, TIS7 and SKMc15 contributed to this phenotype and we could see an additive effect following their deletion (Figure 2c).

Adipocyte differentiation in dKO mice is inhibited due to up regulated DLK-1 levels.

A possible explanation of the lean phenotype of dKO mice was that TIS7 and SKMc15 regulate adipocyte differentiation. Primary MEFs derived from totipotent cells of early mouse mammalian embryos are capable of differentiating into adipocytes and they are versatile models to study adipogenesis as well as mechanisms related to obesity such as genes, transcription factors and signaling pathways implicated in the adipogenesis process³⁵. To test whether TIS7 and SKMc15 are required for adipogenesis we treated MEFs derived from WT, SKMc15 and TIS7 single and dKO mice using an established adipocyte differentiation protocol³⁶. Expression levels of both TIS7 and SKMc15 mRNA increased during the adipocyte differentiation of WT MEFs. TIS7 expression reached maximum levels representing 5.7-fold increase compared to proliferating WT MEFs on day 3 and SKMc15 reached on day 5 the maximum of 2.5-fold expression levels of proliferating

MEFs (Supplementary figure 2). These data suggested that both proteins play a regulatory role in adipogenesis, however differ in their mechanisms and timing. Eight days after initiation of adipocyte differentiation a remarkable reduction of adipocyte differentiation potential in SKMc15, TIS7 KO MEFs and dKO MEFs was observed, as characterized by the formation of lipid droplets stained by oil red O (Figure 3a). Quantification of this staining revealed that fat vacuole formation in cells derived from SKMc15, TIS7 and dKO mice represented 65 %, 35 % and 24 % of the WT cells, respectively (Figure 3b). These data indicated that both, TIS7 and SKMc15 were critical for adipocyte differentiation and that the defect in adipogenesis could be responsible for the resistance of dKO mice to HFD-induced obesity.

Wnt/ β -catenin signaling is an important regulatory pathway for adipocyte differentiation ³⁷. Consistently, others and we have shown that TIS7 deficiency causes up regulation of Wnt/ β -catenin transcriptional activity. Therefore, we have assessed Wnt signaling activity in MEFs by measuring transcriptional activity using TOPflash, TCF-binding luciferase reporter assays. As shown in Figure 3c, Wnt signaling activity, when compared to the WT MEFs, was not regulated in SKMc15 KO, but significantly up regulated in TIS7 KO ($P<0.0001$) and also in dKO MEFs ($P<0.01$). Supporting these findings, Western blot analyses of WAT samples also identified increased β -catenin protein levels in WAT of TIS7 KO and in dKO mice ($P<0.0001$), but not in SKMc15 KO mice (Figure 3d, top panel) suggesting the involvement of TIS7 but not SKMc15 in the Wnt signaling pathway regulation.

Next, we analyzed the expression levels of DLK-1, a negative regulator of adipogenesis and at the same time known target of Wnt signaling ³¹. DLK-1 protein was significantly upregulated in WAT isolated from TIS7 and SKMc15 KO as well as from dKO mice (Figure 3d, bottom panel). The qPCR analysis revealed a significant up regulation of DLK-1 mRNA levels in dKO WAT samples ($P<0.01$) and similarly in dKO MEFs ($P<0.05$) when compared to the WT controls (Figure 4a). Moreover, whereas in the WT MEFs the expression of DLK-1 was strongly up regulated only during the first day of adipocyte differentiation and then over the next days declined to basal levels, in dKO MEFs DLK-1 was up regulated ($P<0.001$) throughout the entire 8 days of differentiation (Figure 4b). This result was confirmed on the protein level by Western blot analyses of

lysates from 8 day differentiated adipocytes (Figure 4c). A rescue experiment was conducted to confirm that DLK-1 expression was TIS7- and SKMc15-dependent. dKO MEFs were transiently co-transfected with TIS7 and SKMc15 vectors and 48 hours later the mRNA expressions were analyzed by RT-qPCR. Ectopic co-expression of TIS7 and SKMc15 significantly ($P<0.05$) down regulated the DLK-1 expression (Figure 4d). Accordingly, TIS7 and/or SKMc15 are involved in the regulation of DLK-1 expression, but the molecular mechanism remained unclear.

Our earlier study revealed that TIS7 binds directly to DNA and via interaction with PRMT5 regulates gene expression¹⁴. Therefore, we performed chromatin immunoprecipitation (ChIP) experiments where we studied the binding of TIS7 and SKMc15 as well as known transcription factors (β -catenin and H4R3me2s (symmetrically dimethylated histone H4 at arginine residue 3))³¹ to the regulatory elements of the DLK-1 gene. In dKO MEFs treated 8 days with the adipocyte differentiation cocktail we found increased β -catenin binding to the β -catenin/TCF binding site 2 of the Dlk-1 regulatory element when compared to WT MEFs (Figure 4e). On the other hand, binding of H4R3me2s to the same Dlk-1 regulatory element was significantly reduced ($P<0.0001$). We could not identify any direct binding of TIS7 or SKMc15 proteins to two different DLK-1 regulatory elements (Dlk-1 region A and β -cat/TCFbs2), neither in WT nor in dKO MEFs, suggesting rather an epigenetic regulation than *via* their direct binding to DLK-1 regulatory elements. We concluded that TIS7 and SKMc15 are required to restrain the DLK-1 levels, through the Wnt/ β -catenin signaling pathway and yet another so far unknown mechanism.

In a search for a SKMc15-specific regulatory mechanism of Dlk-1 levels we focused on the translational regulation since previously was shown that general reduction of protein synthesis and downregulation of the expression and translational efficiency of ribosomal proteins are events crucial for the regulation of adipocyte differentiation¹⁷. Interestingly, IFRD2 (SKMc15) was recently identified as a novel specific factor capable of translationally inactivating ribosomes¹⁶. Because SKMc15 may play a crucial role in the adipogenesis regulation we tested in our following experiment the effect of SKMc15 knockout on the general translational efficiency of WT, TIS7, SKMc15 single and double KO MEFs. Cells were incubated 30 minutes in the absence

of methionine and cysteine, followed by one hour in the presence of ³⁵S-methionine. As shown in Figure 5a, there was a significant increase in the general translational activity of MEFs lacking SKMc15 alone or both TIS7 and SKMc15, but not in TIS7 single knockout cells. Therefore, we concluded that SKMc15 alone, but not TIS7 inhibits the general translational activity necessary for the induction of adipogenic differentiation, also via the translational regulation of Dlk-1. This finding supported the result shown in Figure 3d where the DLK-1 protein levels were significantly induced in WAT samples isolated from SKMc15 single knockout mice.

TIS7 and SKMc15 regulate adipocyte differentiation through DLK-1, MEK/ERK pathway, PPAR γ and C/EBP α .

DLK-1 protein carries a protease cleavage site in its extracellular domain ³⁸ and is secreted. The extracellular domain of DLK-1 is cleaved by ADAM17, TNF- α Converting Enzyme to generate the biologically active soluble DLK-1 ¹⁹. DLK-1 mRNA and protein levels are high in preadipocytes but DLK-1 expression is absent in mature adipocytes. Hence, adding soluble DLK-1 to the medium inhibits adipogenesis ³⁹. To test if the dKO MEFs secreted DLK-1, cell culture media from MEFs treated 8 days with the adipocyte differentiation cocktail were collected and analyzed by Western blotting. As shown in Figure 5b, dKO cells secreted DLK-1 protein, but in an identical volume of the cell culture medium from WT MEFs no DLK-1 could be detected. To prove that secreted DLK-1 could inhibit adipocyte differentiation of dKO MEFs, we cultured WT MEFs with conditioned medium from dKO MEFs. Adipocyte differentiation of WT MEFs was strongly inhibited by the dKO MEFs-conditioned medium when compared to the control WT cells (Figure 5c, quantified in Figure 5d). Based on these results we concluded that cells derived from dKO mice express increased DLK-1 levels and secreted, soluble DLK-1 may inhibit adipocyte differentiation *in vivo*.

Previous studies showed that soluble DLK-1 activates MEK/ERK signaling, which is required for inhibition of adipogenesis ²⁰. As DLK-1 was strongly up regulated in dKO MEFs during adipocyte differentiation we subsequently analyzed possible activation of the MEK/ERK pathway (Figure 6a). The phosphorylation of p44 and of p42 was up regulated 5-fold and 3-fold (P<0.05), respectively, in the adipocyte-differentiated dKO

when compared to the WT MEFs (Figure 6b). Previously, activation of MEK/ERK by DLK-1 was shown to up regulate the expression of the transcription factor SOX9, resulting in the inhibition of adipogenesis³³. Therefore, we measured SOX9 mRNA expression by RT qPCR in 8 days adipocyte-differentiated MEFs. SOX9 expression was significantly ($P<0.001$) up regulated in 8 days adipocyte-differentiated dKO when compared to WT MEFs (Figure 6c).

The adipocyte differentiation deficiency of dKO MEFs suggested that C/EBP α and PPAR γ might also be regulated through TIS7 and/or SKMc15. It was previously shown that elevated levels of the cleaved ectodomain of DLK1 have been correlated with reduced expression of PPAR γ ⁴⁰. Therefore, we analyzed the differences in PPAR γ expression between WT and dKO MEFs during their differentiation into adipocytes. While PPAR γ expression was strongly induced during adipocyte differentiation from WT MEFs this was barely detectable in dKO MEFs (Figure 7a) and also C/EBP α expression was never induced up to the levels of WT (Figure 7b). In a rescue experiment we showed that the co-expression of TIS7 and SKMc15 in undifferentiated MEFs strongly increased the expression of the PPAR γ in dKO MEFs ($P<0.001$), almost up to the levels of WT MEFs (Figure 7c). The expression of C/EBP α was also strongly up regulated by the co-expression of TIS7 and SKMc15 ($P<0.01$) (Figure 7d). Based on these results we concluded that TIS7 and SKMc15 regulate the expression of both PPAR γ and C/EBP α , both crucial for adipocyte differentiation.

TIS7 and SKMc15 regulate the expression of the lipid transporters CD36 and DGAT1.

PPAR γ induces the expression of CD36, a very long chain fatty acids (VLCFA) transporter in heart, skeletal muscle, and also adipose tissues⁴¹. The regulation of CD36 by PPAR γ contributes to the control of blood lipids. Interestingly, CD36 null mice exhibit elevated circulating LCFA and triglycerides levels consistent with the phenotype of dKO mice and CD36 deficiency partially protected from HFD-induced insulin resistance⁴². Because of down regulated PPAR γ levels in adipose tissues of dKO mice we decided to study in more detail the regulation of CD36 in these as well. We have found strongly reduced CD36 mRNA expression levels in WAT from dKO mice (64 % of the WT values) (Figure 8a). Next, we analyzed CD36 in WT and dKO MEFs

before onset and during adipocyte differentiation. As long as CD36 mRNA expression substantially increased in WT MEFs, there were almost undetectable transcript levels of CD36 in dKO cells treated with the adipocyte differentiation cocktail (Figure 8b). Diacylglycerol acyltransferase 1 (DGAT1), a protein associated with the enterocytic triglyceride absorption and intracellular lipid processing [83] is besides CD36 another target gene of adipogenesis master regulator PPAR γ ⁴³. DGAT1 mRNA levels are strongly up regulated during adipocyte differentiation⁴⁴, its promoter region contains a PPAR γ binding site⁴⁵ and DGAT1 is also negatively regulated by the MEK/ERK pathway⁴⁶. DGAT1 expression was shown to be increased in TIS7 transgenic mice³ and its expression was decreased in the gut of high fat diet-fed TIS7 KO mice⁸. Importantly, DGAT1 expression in adipocytes and WAT is up regulated by PPAR γ activation⁴³. Therefore, we measured DGAT1 mRNA levels during the differentiation of WT and dKO MEFs into adipocytes to analyze the role of TIS7 and SKMc15 on the regulation of this protein involved in adipogenesis and triglyceride processing. As long as DGAT1 expression substantially increased during the differentiation of WT MEFs, there was no difference in DGAT1 mRNA levels in dKO cells treated with the adipocyte differentiation cocktail (Figure 8c). Therefore, we concluded that both, TIS7 and SKMc15 regulate expression of multiple proteins involved in adipocyte differentiation and function, thereby contributing to the lean phenotype of dKO mice.

Discussion

In this study we show that simultaneous depletion of TIS7 and of its orthologue SKMc15, a protein recently identified as a translational inhibitor, caused severe reduction of adipose tissues and resistance against high fat-induced obesity in mice. We identified two parallel mechanisms leading to a deficiency in adipocyte differentiation. Firstly, TIS7-regulated Wnt signaling affected Dlk-1 transcription and secondly, our study provided the first experimental evidence for SKMc15 acting as a translational inhibitor that among other proteins might control DLK-1 protein levels, thereby also contributing to the regulation of adipogenesis (Figure 9).

dKO mice were phenotypically similar with both, CD36 deficient and DLK-1 transgenic mice, namely in decreased amounts of WAT and resistance to HFD-induced obesity. Previous studies showed that overexpression of TIS7 caused increased intestinal lipid transport resulting in elevated body weight gain during HFD feeding ³⁶. Knockout of TIS7 and SKMc15 impaired absorption of free fatty acids from the lumen into enterocytes and reduced rate of fat absorption from intestines into the circulation. Simultaneously, higher concentrations of free fatty acids in the feces of dKO mice suggested that mice lacking TIS7 and SKMc15 suffer from an intestinal lipid uptake deficiency, yet another contributing reason for the lean phenotype of these mice (data not shown). In dKO mice we found inhibited PPAR γ and C/EBP α levels, induced MEK/ERK pathway, and decreased expression of CD36 and DGAT1, all hallmarks of up regulated DLK-1, a known adipogenesis inhibitor. TIS7 and SKMc15 acted after the commitment to the preadipocyte stage since the elevated DLK-1 and β -catenin levels found in WAT of dKO mice were characteristic for the preadipocyte stage ⁴⁷.

It is known that disruption of Wnt signaling in embryonic fibroblasts results in spontaneous adipocyte differentiation ⁴⁸ and opposite, stabilized β -catenin keeps cells in the preadipocyte stage ²³. Consistent with this knowledge we found up regulated β -catenin protein levels in WAT of dKO mice and increased Wnt signaling activity in dKO MEFs. It has to be mentioned that our data were different to those by Nakamura *et al.* who showed that following the TIS7 overexpression in adipocytes was up regulated Wnt/ β -catenin signaling and inhibited oil red O staining ⁵. However, one has to take into consideration the difference in cell systems used in these two studies. All results presented in Nakamura's study were obtained in 3T3-L1 cells fibroblasts following overexpression of TIS7. Opposite to that, we have studied the role of endogenous TIS7 in cells derived from wild type or knockout mice. Moreover, we have found ubiquitous TIS7 expression in all WT mouse organs without any pre-treatment such as e.g. hypoxia. On the other hand, Nakamura *et al.* identified up regulated TIS7 expression levels in WAT of obesity model mice. This result, however, fully supports our findings of lean phenotype in TIS7 and SKMc15 dKO mice.

322 We demonstrated that in dKO MEFs, induced for adipocyte differentiation, the increase in Wnt signaling led
323 to up regulated binding of β -catenin to the DLK-1 gene regulatory elements, resulting in sustained DLK-1
324 expression. In adipogenesis DLK-1 expression is down regulated through histone methylation by COPR5
325 and PRMT5 that prevent β -catenin binding to the DLK-1 gene ³¹. Interestingly, in dKO MEFs we found weaker
326 binding of dimethylated H4R3 to the DLK-1 gene that was consistent with our previous findings on the
327 negative role of TIS7 in epigenetic regulation of gene expression including the PRMT5 activity ¹⁴.

328 There was a difference in effects of ectopic expression of TIS7, SKMc15 and their co-expression on DLK-1
329 levels (data not shown), confirming the hypothesis TIS7 and SKMc15 regulate DLK-1 levels via two
330 independent pathways/mechanisms. Despite the fact that SKMc15 knockout had no effect on Wnt signaling
331 it nevertheless affected DLK-1 levels, suggesting a contribution of SKMc15 to its regulation. Since SKMc15
332 was identified as a novel factor translationally inactivating ribosomes ¹⁶ and since down regulation of the
333 protein synthesis machinery is an essential regulatory event during early adipogenic differentiation ¹⁷, we
334 hypothesize that SKMc15 regulates DLK-1 levels through translational regulation, however additional
335 investigations will be necessary to confirm this mechanism. Elevated DLK-1 levels in dKO MEFs activated
336 the MEK/ERK pathway thereby decreasing PPAR γ and C/EBP α levels important for adipogenic
337 differentiation. Besides that, the expression of SOX9 was up regulated, suggesting that the dKO MEFs keep
338 their proliferative state and cannot enter differentiation into mature adipocytes ²⁰.

339 Down regulation of PPAR γ and C/EBP α implied changes in expression of downstream adipogenesis-related
340 genes. Among them, decreased CD36 and DGAT1 levels were a plausible explanation of the dKO mice lean
341 phenotype since it is known that both proteins play a functional role in differentiation of murine adipocytes
342 and their deficiency impairs fat pad formation independent of lipid uptake ⁴⁹. Our findings implicate that TIS7
343 and SKMc15 play a up to now unknown role in the regulation of CD36 and therefore possibly contribute to
344 CD36 related pathogenesis of human metabolic diseases, such as hypoglycemia ⁵⁰, hypertriglyceridemia ⁵¹,
345 and disordered fatty acid metabolism ^{52, 53}.

We and others have previously shown that TIS7 is involved under both, chow and HFD conditions in intestinal triglyceride absorption. Here we show for the first time the role of TIS7 and its orthologue SKMc15 in adipocyte differentiation. Moreover, our data presented here document the physiological function of SKMc15 as a translational inhibitor. To our surprise, although TIS7 and SKMc15 share high sequence homology and a functional role in the adipogenesis, they use two independent regulatory mechanisms.

Methods

Generation of animal models. All animal experiments were performed in accordance with Austrian legislation BGB1 Nr. 501/1988 i.d.F. 162/2005).

Mice lacking TIS7 were previously described ⁴. SKMc15 KO mice generation: targeting construct contained SKMc15 gene locus exons. A loxP site and a neomycin resistance gene inserted at position 105515 (AY162905). The neomycin cassette was flanked by two frt sites. A second loxP site was inserted at position 102082 (AY162905). Further downstream, 15 additional nucleotides, part of intron 1, exon 2 (splice site: donor and acceptor) and the CDS of hrGFP from the Vitality hrGFP mammalian expression vector pIRES-hrGFP-2a (Stratagene) were added. The targeted construct was electroporated into Sv129 mouse ES cells. After the selection with G418 single cell clones were screened by PCR and confirmed by Southern blot analysis. After Cre recombinase treatment cell clones were screened by PCR. Clones with floxed gene deleted, were used for blastocyst injection into C57Bl/6J mice. Two male chimeras with $\geq 40\%$ or more agouti coat color were mated to C57Bl6 females. The knockout mouse strain was derived from one male mouse carrying the allele of interest. Heterozygous mice were back-crossed to C57Bl6 mice for 9 generations. dKO mice were generated by crossing the SKMc15 KO mice with the TIS7 single KO mice. The resulting mice were screened by PCR and double heterozygous mice used for further breeding until homozygosity. In order to achieve maximal homogeneity of experimental groups, in all experiments presented here we used only male mice.

SKMc15 knockout Southern blot analysis. XbaI restriction sites (position 109042; position 102079; position 92253) located in the SKMc15 locus (AY162905). Fragment detected by the Southern probe: 9.6 kb wt (Supplementary figure 3a, insert, band V); 15 kb in deleted locus (band IV). The 566 bp long probe for hybridization from genomic DNA (96605 – 97171; AY162905). PCR primer sequences: RK 150 Fwd: 5'-GGTCCTGCCACTAATGCACTG-3'; RK 151 Rev: 5'-GCAGACAGATGCCAGGAAGAC-3'.

SKMc15 knockout PCR genotyping analysis. hrGFP insert in the 3' UTR detected by primer GFP2 5'-AGCCATACCACATTTGTAGAG-3' and RK101 3' UTR (5'-TGATGATAGCTTCAAAGAGAA-3'; 100617 – 100591 of the SKMc15 locus (AY162905). PCR product 1700 bp. SKMc15 detection: RG1; 5'-TGTGGCCTTTATCCTGAGTC-3'; 102286–102266) and RG2; 5'-TGGCTTCATTTACACTACTCCTT-3'; 101860 – 101882 primers (Supplementary figure 3a). WT allele PCR product 426 bp and the targeted allele PCR product 1772 bp (Supplementary figure 3b). TIS7 genotype tested as explained previously⁴ (Supplementary figure 3c).

Growth monitoring and body composition measurement. Mice weaned at 3 weeks, regular chow diet, weighed weekly. Dual energy X-ray absorptiometry was measured with Norland scanner (Fisher Biomedical). Micro-CT experiments performed using vivaCT 40 (Scanco Medical AG). The scans were performed using 250 projections with 1024 samples, resulting in a 38µm isotropic resolution. Tube settings: 45kV voltage, 177µA current, integration time 300ms per projection. Image matrix 1024*1024 voxels and a grayscale depth of 16 bit. The length of the image stack was individually dependent, starting from the cranial end of the first lumbar vertebrae, to the caudal end of the fifth lumbar vertebrae. The image reconstruction and post processing were performed using the Scanco Medical system software V6.6. For the adipose tissue evaluation an IPL (image processing language) script by Judex et al., provided by Scanco medical AG was modified to the scanner individual parameters leading to 2 values lower threshold than in the original script for the adipose tissue filters 76. The script calculated the total abdominal volume without potential air in the

cavities. A separation of subcutaneous and visceral fat mass was used only for visualization. For the quantitative fat mass analysis we used 56 male 5 to 12 month old mice (mean age 9.35 ± 2.03 month) reflecting an adult to mid aged cohort defined by Flurkey et al. 2007⁵⁴. The development of adipose tissue in healthy mice is stable between 4 and 12 months⁵⁵. Therefore, sex and age could have only very limited influence on the experimental results. For the quantitative comparison, the percent contribution of the abdominal fat to the body weight was calculated using a mean weight of 0.9196 g/ml for adipose tissue⁵⁴. Statistics were performed using an ANCOVA with a Bonferroni corrected Post Hoc testing on the μ CT fat data and the body mass data.

Metabolic measurements. The indirect calorimetry trial monitoring gas exchange, activity, and food intake was conducted over 21 hours (PhenoMaster TSE Systems). Body mass and rectal body temperature before and after the trial were measured. The genotype effects were statistically analyzed using 1-way ANOVA. Food intake and energy expenditure were analyzed using a linear model including body mass as a co-variate.

HFD feeding. Age-matched (7-week-old) male TIS7^{-/-} SKMc15^{-/-} and WT mice were caged individually and maintained up to 3 weeks on a synthetic, high saturated fat (HFD) diet (TD.88137; Ssniff). Animals were weighted every 4th day between 08:00 and 10:00. Small intestines were harvested for oil red O staining to detect lipid accumulation. Intestines, liver, muscles and adipose tissue were collected for total RNA and protein isolation. Unfixed intestines were flushed with PBS using a syringe, embedded in Tissue-Tek (Sakura, 4583) and frozen in liquid nitrogen for immunohistochemical analysis.

Quantitative food consumption and fecal fat determination. Adult mice were acclimatized to individual caging and to the HFD for a week, monitored for weight gain and their food intake daily. The daily food intake data were pooled for the following 7 days and the food intake was estimated (g/day). Feces were collected daily and weighted for 7 days after second week of the HFD consumption.

421
 422 *Antibodies, viral and cDNA constructs.* Antibodies: anti-CD36 AbD Serotec (MCA2748), Abcam (ab36977),
 423 p44/42 MAPK (Erk1/2) and Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Cell Signaling Technology
 424 (9102, 9101), β -catenin antibody Sigma (C2206), anti-DLK Abcam (ab119930), anti-histone H4R3me2s
 425 antibody Active Motif (61187). For ChIP experiments were used anti-TIS7 ¹¹ and anti-SKMc15 ⁴ rabbit
 426 polyclonal antibodies previously proven for ChIP suitability in ¹⁴. pTOPflash reporter construct was a gift from
 427 H. Clevers (University of Utrecht, Holland). TIS7 construct was described previously ¹¹. Partial cds of
 428 mSKMc15 was amplified by PCR and cloned into pcDNA3.1(-)/MycHis6 (Invitrogen).

429
 430 *Cell culture and adipocyte differentiation.* MEFs were generated from 16-day old embryos. After dissection
 431 of head for genotyping, removal of limbs, liver and visceral organs embryos were minced and incubated in 1
 432 mg/mL collagenase (Sigma-Aldrich, C2674) 30 mins at 37°C. Embryonic fibroblasts were maintained in
 433 growth medium containing DMEM high glucose (4.5 g/l); sodium pyruvate, L-glutamine, 10% FCS (Invitrogen,
 434 41966029), 10% penicillin/streptomycin at 37°C in 5% CO₂. Adipocyte differentiation treatment was medium
 435 with 0.5 mM 3-isobutyl-1-methylxanthine (I5879), 1 μ M dexamethasone (D4902), 5 μ g/ml insulin (I2643) and
 436 1 μ M Rosiglitazone (Sigma-Aldrich, R2408). After 3 days growth medium containing 1 μ g/ml insulin cells
 437 were differentiated for 8 days. To visualize lipid accumulation adipocytes were washed with PBS, fixed with
 438 6 % formaldehyde overnight, incubated with 60 % isopropanol, air dried and then incubated with oil red O.
 439 Microscopic analysis was followed by isopropanol elution and absorbance measurement at 490 nm.

440
 441 *Analysis of translation by metabolic labelling.* Pulse labelling of mitochondrial proteins was performed as
 442 described before ⁵⁶, with the following changes: 1x 10⁶ wild-type and dKO MEFs cells per plate were seeded
 443 and cultivated for 24 h. Cells were washed twice with PBS followed by one wash with labelling medium (ESC
 444 medium without cysteine and methionine) and a 30 min incubation in labelling medium. To label newly
 445 translated products, 200 μ Ci of [³⁵S]-methionine (10 mCi/ml; Hartman Analytic) were added and the cells

were incubated for 1 h. Cells were washed and incubated another 10 min in standard medium at 37 °C. Cells were then harvested by trypsinizing, washed once with ice-cold PBS, extracted with ice-cold RIPA buffer containing protease-inhibitors and briefly sonicated. Proteins were fractionated by gel electrophoresis in 16% Tricine gels (Invitrogen) and stained with Coomassie brilliant blue, and radioactive signals were visualized by phosphorimaging. Signal intensities were quantified using the Image Studio Lite (v5.2) software.

RT-PCR. Tissues from animals fed for 3 weeks with HFD were snap frozen and stored at –80°C. Total RNA was isolated using the TRIzol reagent (Invitrogen, 15596026). RNA was then chloroform-extracted and precipitated with isopropanol. The yield and purity of RNA was determined by spectroscopic analysis; RNA was stored at –80°C until use.

Quantitative RT-PCR and statistics. Total RNA were treated with DNase1 and reverse transcribed to cDNA by Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622) with oligo dT primers. Quantitative RT-PCR was performed using Taqman probes and primer sets (Applied Biosystems) specific for CD36 (assay ID Mm00432398_m1), Dgat1 (Mm00515643_m1), Pparγ (Mm00440940_m1), C/EBPα (Mm00514283_s1), DLK-1 (Mm00494477_m1) and Sox9 (Mm00448840_m1). Ribosomal protein 20 (assay ID Mm02342828_g1) was used as normalization control for quantification by the ddCt method. PCR reactions were performed using 10 µl cDNA in PikoReal 96 real-time PCR system (Thermo Scientific). Quantification data were analyzed by 2-tailed, type 2 Student's t test.

Transient transfections and luciferase assay. pGL2-Basic (Promega, E1641) or pGLCD36⁵⁷ were used as reporter constructs. Expression constructs or empty vector DNA as a control were co-transfected. pCMV-β-Gal plasmid was used to normalize for transfection efficiency. For luciferase reporter assays, 1.5x10⁵ cells were seeded into 24-well plates and transfected after 24 hours with indicated plasmid combinations using Lipofectamine Plus™ Reagent (Invitrogen, 15338030). The total amount of transfected DNA (2 µg DNA per

well) was equalized by addition of empty vector DNA. Cells were harvested 48 h post-transfection in 0.25 M Tris, pH 7.5, 1% Triton X-100 buffer and assayed for both luciferase and β -galactosidase activities. Luciferase activity and β -galactosidase activity were assayed in parallel by using the Lucy 2® detection system (Anthos). Transfections were performed in triplicates and all experiments were repeated several times.

Chromatin immunoprecipitation (ChIP). Chromatin was isolated from TIS7 WT and dKO formaldehyde-treated, 8 days adipocyte differentiated MEFs using the EpiSeeker Chromatin Extraction Kit (Abcam, ab117152). ChIP analyses were carried out as described previously⁵⁸. Sequence of the oligonucleotides for two regions of the Dlk-1 promoter, encompassing TCF and β -catenin binding sites were as defined in³¹. Sonicated chromatin was centrifuged at 15.000x g for 10 min at 4°C, and the supernatant (65 μ g of sheared DNA per each IP) was diluted ten-fold with cold ChIP dilution buffer containing 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, 0.01% (w/v) SDS, 1.1% (w/v) Triton X-100 and 1.2 mM EDTA with protease inhibitors. Samples were pre-cleared for 1 h with protein A Sepharose CL-4B (Sigma-Aldrich, 17-0780-01) beads blocked with 0.2 μ g/ μ l sonicated herring sperm DNA (Thermo Fisher, 15634017) and 0.5 μ g/ μ l BSA (NEB, B9000 S). Immunoprecipitations were performed at 4°C overnight. Immune complexes were collected with protein A Sepharose for 1h at 4°C followed by centrifugation at 1000 rpm and 4°C for 5 min. Beads were washed with 1ml low salt wash buffer (20 mM Tris-HCl pH 8.1, 150 mM NaCl, 0.1%(w/v) SDS, 1%(w/v) Triton X-100 (Merck), 2 mM EDTA), high salt wash buffer (20 mM Tris-HCl pH 8.1, 500 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) Triton X-100, 2 mM EDTA), LiCl wash buffer (10 mM Tris-HCl pH 8.1, 250 mM LiCl, 1% (w/v) sodium deoxycholate, 1% (w/v) IGEPAL-CA630, 1 mM EDTA) for 5 min at 4°C on a rotating wheel, and twice with 1 ml TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Protein-DNA complexes were eluted from antibodies by adding a freshly prepared elution buffer containing 1% SDS and 0.1 M NaHCO₃. The eluate was reverse cross linked by adding NaCl to a final concentration of 0.2 M and incubating at 65 °C for four hours. Afterwards the eluate was treated with Proteinase K at 45 °C for 1 hour. The immunoprecipitated DNA was then isolated by phenol/chloroform precipitation and used as a template for real-time-quantitative PCR. The

primer pairs specific for regulatory regions of the Dlk-1 gene were selected as described before³¹. Reactions with rabbit IgG or with 1.23% of total chromatin (input) were used as controls. For real-time-quantitative PCR a PikoReal System was used. Signals were normalized to input chromatin and shown as % input. The raw cycle threshold (Ct) values of the input were adjusted to 100% by calculating $\text{raw Ct} - \log_2(100/\text{input})$. To calculate the % input of the immunoprecipitations, the equation $100 \times 2^{[\text{Ct (adjusted input to 100\%)} - \text{Ct (IP)}]}$ was applied.

Statistics. Unpaired, 2-tailed Student's t tests were used for all statistical comparisons. A P value of < 0.05 was considered significant.

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Author contributions

I.V., D.C., M.D.E., V.K., J.Ro., M.K., T.V. and L.A.H. designed the study. I.V., D.C., K.P., R.G., J.Ro., M.H., V.K., G.D., F.S., and T.V. performed experiments. I.V., D.C., T.V. and L.A.H. wrote the manuscript.

Figure legends

Figure 1. dKO mice display postnatal growth retardation and less body fat.

a) Growth curves of WT and dKO mice on chow diet (n fluctuate depending on the time point of measurement). b) DEXA measurements of WT and dKO mice. Left panel depicts the absolute values of fat and lean mass per animal and the right panel represents values normalized to the total body weight of animals (n=15). c) Micro-computed tomography measurements identified a lack of abdominal fat in single and dKO mice. 3-dimensional reconstitution of images of the abdominal fat mass distribution in WT and KO mice. Yellow color represents subcutaneous and red visceral fat mass. Black compartments are shadows resulting as part of the lightning model for the 3D volume rendering.

Figure 2. dKO mice are resistant against HFD-induced obesity.

a) Mass contribution [%] of the abdominal fat in correlation to the total body weight [g]. A linear regression is overlaid for each group individually. The regression results for the WT group are $y = 0,342x - 11,13$ with a R^2 of 0,74; for the TIS7 KO group $y = 0,143x - 3,91$ with a R^2 of 0,82; for the SKMc15 KO $y = 0,005x + 0,48$ with a R^2 of 0,04 and for the dKO $y = 0,011 + 0,01$ with a R^2 of 0,1. ANCOVA analysis for the fat mass as a percentage of the body weight as covariant was performed. b) Effect of single SKMc15 or TIS7 knockout and TIS7 SKMc15 double knockout on total body weight in [g] and body fat amount normalized to the body weight in [%]. c) TIS7 and SKMc15 knockout reduced the gain of body weight of mice fed with HFD. 7 weeks old male WT and dKO mice were caged individually and maintained up to 21 days on HFD. Data shown are mean $\pm$ SEM, $n \geq 9$ per genotype, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. Adipocyte differentiation and Wnt signaling pathway are significantly affected in dKO

mice. a) Representative oil red O staining of MEF cells following 8 days of the adipocyte differentiation protocol. Images were cropped and space bars represents always 20 μ m. b) Quantification of oil red O staining from three independent adipocyte differentiation experiments. Student's t-test *** $P < 0.001$. c) Wnt signaling activity was measured in MEFs transiently co-transfected with the Tcf reporter plasmid pTOPflash and β -galactosidase expression vectors. Transfection efficiency was normalized using the β -galactosidase

values. The experiment was repeated three times. Data shown are mean $\pm$ SEM. d) Top panel: Western blot analysis of β -catenin amounts in WAT samples. One representative Western blot from 3 biological repetitions is shown. Bottom panel: DLK-1 is significantly up regulated both in TIS7, SKMc15 single KO and also in dKO mice.

Figure 4. DLK-1 levels are lower and β -catenin binding to the Dlk-1 gene is increased in dKO MEFs.

a) DLK-1 mRNA expression measured by qPCR in WAT and MEFs. Values were normalized on GAPDH, n=3. Error bars indicate standard deviations. b) DLK-1 mRNA expression measured in MEFs treated with the adipocyte differentiation cocktail for given times. WT MEFs values were set as 1. c) DLK-1 protein Western blot analysis in MEFs after 8 days of adipocyte differentiation. Mean of 3 biological repeats; insert is one representative Western blot. d) DLK-1 mRNA levels in dKO MEFs were down regulated following ectopic co-expression of TIS7 and SKMc15. mRNA expression levels were analyzed by RT qPCR 48 hours following the transfection. WT MEFs values were set as 1, n=3; Student's t-test *P<0.05. Error bars indicate standard deviations. e) Recruitment of indicated proteins to regulatory regions of the Dlk-1 promoter in WT and KO MEFs was analyzed by ChIP at day 8 of the adipocyte differentiation. Values are expressed as the percentage of immunoprecipitated chromatin relative to input and are the mean of triplicates. ChIP analysis identified increased specific β -catenin binding to its DLK-1 regulatory element in dKO samples. Pre-immune serum and IgG were used as background controls. n=3, Data shown are mean $\pm$ SEM, Student's t-test ***P < 0.001.

Figure 5. SKMc15 is a translational inhibitor. Up regulated DLK-1 in dKO MEFs inhibits adipocyte

differentiation. a) translational analysis – *in vivo* metabolic staining of MEFs with ³⁵S-methionine. Equal numbers of cells were seeded and 24 hours later treated as explained in Methods section. Identical volumes of cell lysates were separated by SDS-PAGE, gel was dried and analyzed by a phosphorimager. Equal loading was documented by the Coomassie blue staining of the gel. b) dKO MEFs secrete DLK-1 protein into

the cell culture medium. Identical volumes of media from WT and dKO cells 8 days treated with the adipocyte differentiation cocktail were analyzed by Western blot. Equal loading of samples was evaluated by amido black staining of the membrane. c) dKO MEFs-conditioned medium inhibited adipocyte differentiation in WT MEFs. MEFs were treated 8 days with the adipocyte differentiation cocktail. WT MEFs treated with the dKO-conditioned medium showed reduced differentiation. Images were cropped and space bars represent always 20µm. d) Quantification of the oil red O staining of samples shown in panel C.

Figure 6. MAPK signaling and Sox9 expression are induced in dKO MEFs.

a) Representative Western blots of phospho-p44 and phospho-p42 from 8 days adipocyte differentiation cocktail-treated MEFs. b) Normalization on p44 and p42; n=3. Error bars indicate standard deviations. WT values were set as 100 %. c) SOX9 mRNA levels were up regulated in dKO MEFs. SOX9 expression was measured by qPCR in 8 days adipocyte-differentiated WT and dKO MEFs. SOX9 values were normalized on GAPDH expression. WT MEFs values were set as 1, n=3. Data shown are mean ± SEM. Student's t-test **P < 0.01.

Figure 7. Adipocyte differentiation regulatory genes are down regulated in dKO mice.

a) PPARγ and b) C/EBPα mRNA levels were down regulated in dKO adipocyte-differentiated MEFs. Gene expression was measured by qPCR during the treatment with the adipocyte differentiation cocktail. Values were normalized on GAPDH expression. WT MEFs values were set as 1, n=3. Data shown are mean ± SEM. c) Ectopic co-expression of TIS7 and SKMc15 in undifferentiated MEFs significantly increased levels of adipogenic genes PPARγ and d) C/EBPα. Student's t-test **P < 0.01, ***P < 0.001.

Figure 8. Target genes of adipocyte differentiation regulatory genes are down regulated in dKO mice.

a) qPCR analysis of CD36 mRNA levels in WAT, *P< 0.05 b) qPCR analysis of CD36 in MEFs differentiating into adipocytes. Relative expression levels were normalized on GAPDH expression. WT

MEFs values were set as 1. c) DGAT1 mRNA levels were down regulated in dKO adipocyte-differentiated MEFs, ***P < 0.001.

Figure 9. Proposed model of TIS7 and SKMc15 molecular mechanisms of action during adipocyte differentiation.

Two parallel mechanisms leading to a deficiency in adipocyte differentiation: TIS7-regulated Wnt signaling affected Dlk-1 transcription and SKMc15 acting as a translational inhibitor also contributed to the regulation of adipogenesis.

Conflict of interest.

The authors declare that they have no conflict of interest.

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Vietor_Figure 2

Vietor_Figure 3

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Vietor_Figure 7

Vietor_Figure 8

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Vietor_Figure 9