TRAIL (TNF-related apoptosis-inducing ligand) inhibits human adipocyte differentiation via caspase-mediated downregulation of adipogenic transcription factors

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Tumor necrosis factor-α (TNFα) and other ligands of the TNF superfamily are potent regulators of adipose tissue metabolism and play a crucial role in the obesity-induced inflammation of adipose tissue. Adipose tissue expression levels of TRAIL (TNF-related apoptosis-inducing ligand) and its receptor were shown to be upregulated by overfeeding and decreased by fasting in mice. In the present study we aimed to elucidate the impact of TRAIL on adipogenesis. To this end, human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes as well as stromal-vascular cells isolated from human white adipose tissue were used as model systems. Human recombinant TRAIL inhibited adipogenic differentiation in a dose-dependent manner. It activated the cleavage of caspase-8 and -3, which in turn resulted in a downregulation of the key adipogenic transcription factors C/EBPα, C/EBPγ, and PPARδ. The effect was completely blocked by pharmacological or genetic inhibition of caspases. Taken together we discovered a so far unrecognized function of TRAIL in the regulation of adipogenesis. Targeting the TRAIL/TRAIL receptor system might provide a novel strategy to interfere with adipose tissue homeostasis.

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TRAIL was first discovered as an apoptosis-inducing factor. It became famous as a potential anticancer agent because it seemed to selectively induce apoptosis in cancer cells. Although preclinical studies were promising, clinical trials applying either human recombinant TRAIL or agonistic antibodies revealed only a limited therapeutic benefit (summary of clinical studies in Lemke et al.9). Therefore, current efforts focus on the identification of compounds, which potently and selectively sensitize cancer cells to TRAIL-induced apoptosis.9

Recently, TRAIL is increasingly studied in the context of nonmalignant diseases, specifically metabolic diseases such as obesity, type 2 diabetes mellitus, and cardiovascular disease.10,11 Choi et al.12 demonstrated that circulating TRAIL levels correlate positively with the body mass index (BMI) and serum lipid levels. Accordingly, Brombo et al.13 found a significant correlation of serum TRAIL with waist circumference and with LDL-cholesterol. In an experimental setup of physical inactivity, serum TRAIL levels increased with overfeeding, but decreased upon caloric restriction in healthy subjects.14 This is in line with our finding that the adipose tissue mRNA expression of TRAIL robustly decreased upon fasting in C57/BL6 mice and rapidly increased again after re-feeding.15

Despite TRAIL receptor expression, both preadipocytes and adipocytes are resistant to TRAIL-induced apoptosis.16,17
Instead, TRAIL exerts several non-apoptotic functions in the context of adipose tissue. For example, it regulates insulin-stimulated metabolic processes such as glucose uptake in a caspase-dependent manner. Moreover, TRAIL was identified as a potent proliferative factor in preadipocytes. Not only the pool of precursor cells but also the number of mature adipocytes represent important regulators of adipose tissue homeostasis. Therefore, the aim of the current study was to elucidate the impact of TRAIL on adipogenic differentiation.

Results

TRAIL inhibits adipogenic differentiation of human SGBS cells and human primary stromal-vascular cells. To study the effect of TRAIL on adipogenic differentiation, we used human Simpson-Golabi-Behmel syndrome (SGBS) cells, which express both TRAIL-R1 and TRAIL-R2, but show a very low sensitivity to TRAIL-induced apoptosis. We incubated SGBS cells with increasing doses of TRAIL during the first 4 days of the adipogenesis protocol. Already at a concentration of 10 ng/ml a clear decrease in intracellular lipid droplet formation was visible when cells were stained with the lipophilic dye Oil Red O on day 10 (Figure 1a). Morphological evaluation revealed a reduced rate of adipogenic differentiation (Figure 1b). Likewise, the triglyceride content was significantly reduced in a dose-dependent manner (Figure 1c). These changes were accompanied by a robust downregulation of the adipogenic marker genes PPARγ, Glut-4, and adiponectin on the mRNA (Figure 1d) and the protein level (Figure 1e). Importantly, TRAIL also inhibited adipogenic differentiation in human primary stromal-vascular cells isolated from subcutaneous adipose tissue samples of five healthy donors (Figures 1f and g).

The antiadipogenic effect of TRAIL is mediated via TRAIL-R2. To elucidate which TRAIL receptor mediates this antiadipogenic effect, we used agonistic antibodies specific for either TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab). These compounds are currently tested for their anticancer activity in phase I/II studies. Adipogenic differentiation was not affected when TRAIL-R1 was stimulated with mapatumumab. However, targeting TRAIL-R2 with lextatumumab resulted in a clear inhibition of adipogenesis (Figures 2a and b), indicating that TRAIL-R2 is responsible for mediating the antiadipogenic effect of TRAIL.

Non-canonical signaling is not involved in mediating the antiadipogenic effect of TRAIL. TRAIL mediates many of its non-apoptotic effects by non-canonical signaling routes activating NFκB or kinases such as JNK, p38, Akt, and ERK1/2. We first studied NFκB, because this pathway is known to conduct many of the TNFα-induced effects on adipose tissue. There was no phosphorylation of IkBα with TRAIL, while stimulation with macrophage-conditioned medium, which was performed as a positive control, gave a clear signal (Figure 3a). In line with this, inhibition of the NFκB pathway by the small-molecule inhibitor SC-514 was not able to abrogate the effect of TRAIL on adipogenesis (Supplementary Figure S1). We thus exclude the NFκB pathway as a mediator of the antiadipogenic effect of TRAIL.

We observed a slight increase in JNK phosphorylation at the beginning of adipogenic differentiation, which did not differ between control and TRAIL-treated cells. This is most likely caused by the change of media upon adipogenic induction. There was no induction of p38 at all, while the positive control displayed a strong signal. Akt was phosphorylated at any investigated time point with no difference between control and TRAIL-treated cells. In contrast, we detected a strong and sustainable phosphorylation of ERK1/2 upon TRAIL treatment starting at 6 h and persisting during the first 24 h of adipogenic differentiation. Densitometric analysis of ERK1/2 phosphorylation is provided in Supplementary Figure S2.

To elucidate if ERK1/2 activation plays a causal role in the antiadipogenic effect of TRAIL, we blocked its phosphorylation by targeting MEK1/2, the specific upstream kinases of ERK1/2, with the small-molecule inhibitor PD98059 (100 μM). This inhibitor blocked both the basal as well as the TRAIL-induced phosphorylation of ERK1/2 (Figure 3b). PD98059 alone had no influence on adipogenic differentiation and it did not modulate the effect of TRAIL on adipogenesis as reflected by comparable differentiation rate and mRNA expression of adipogenic markers genes (Figures 3c and d). Taken together, this set of experiments demonstrates that the antiadipogenic effect of TRAIL is not mediated by the studied non-canonical signaling routes.

Caspase activation is involved in mediating the antiadipogenic effect of TRAIL. Canonically, TRAIL binds to its surface receptors TRAIL-R1 or TRAIL-R2 leading to receptor trimerization, DISC formation, and cleavage of caspsases that can ultimately result in cell death induction. Under the adipogenic conditions chosen, 30 ng/ml TRAIL triggered a rapid cleavage of caspase-8 with the active p18 fragment clearly detectable from 60 min onwards until 6 h (Figure 4a). Cleavage of caspase-3 occurred within the same timeframe with the active p17 fragment being first visible after 60 min, peaking between 2 and 6 h and weakly persisting for 72 h (Figure 4a). An activity assay using a fluorescently labeled Asp-Glu-Val-Asp (DEVD) peptide that contains a caspase cleavage site revealed that this substrate is cleaved in response to TRAIL treatment (Figure 4b).

Despite this, there was only negligible apoptosis induction. During the timeframe of adipogenesis, cultures appeared healthy without morphological signs of apoptosis such as loss of plastic adherence (Figure 5a). Likewise, the total adherent cell number was not different between control and TRAIL-treated cells (Figure 5b). However, we observed that the cellular composition of the cultures was altered. On day 7, approximately 68% of the adherent cells were adipocytes and 32% were preadipocytes in the control cultures. In contrast, only 28% of cells were adipocytes and 72% were preadipocytes in TRAIL-treated cultures (Figure 5c).

In line with the morphological observations, two different types of apoptosis assays revealed only low amounts of apoptosis. Measuring hypodiploid DNA content, the percentage of specific apoptosis upon TRAIL treatment was ~8% on day 4 and ~3% on day 11 (Figure 5d). Comparably, the
percentage of cells with a decreased mitochondrial membrane potential was ~3% on day 4 and ~1% on day 11 (Figure 5e).

As a positive control, we treated adipocytes on day 4 or day 11 with TRAIL in combination with cycloheximide (CHX), which led to the induction of apoptosis in >30% of the cells (Figures 5d and e). In the TRAIL and CHX-treated apoptotic cultures we observed the cleavage of poly(ADP-ribose)-polymerase (PARP) and lamin A/C, whereas this was not seen upon TRAIL treatment alone (Figure 5f).

This set of experiments demonstrates that there is only a weak, negligible induction of apoptosis upon TRAIL treatment under the chosen experimental conditions, which is in agreement with our previous observations that human preadipocytes and adipocytes are resistant to death ligand-induced apoptosis.16,17 We conclude that a reduction in cell numbers is not responsible for the inhibitory effect of TRAIL on adipogenic differentiation.

To study if caspase activation is a crucial event in the TRAIL-induced inhibition of adipogenesis, we took advantage of a pan-caspase inhibitor. At a concentration of 20 μM zVAD.fmk completely inhibited the TRAIL-induced processing of both caspase-8 and caspase-3 to their fully active fragments (Figure 6a). While the inhibitor alone had no impact on adipogenesis, it completely abrogated the antiadipogenic effect of TRAIL as reflected by the adipogenic differentiation rate (Figure 6b) and adipocyte marker gene expression (Figure 6c). When zVAD.fmk was added after the initial treatment with TRAIL, for example, 12 h later, it had no impact on the antiadipogenic effect of TRAIL (Supplementary Figure S3). We therefore conclude that the relevant
expression of these factors in the presence and absence of TRAIL. We additionally included a treatment with the caspase inhibitor zVAD.fmk to delineate the involvement of caspase activation. On mRNA level, C/EBPδ was only weakly induced upon adipogenic stimulation and TRAIL did not exert any significant regulatory effect (Figure 7a). In contrast, TRAIL clearly inhibited the upregulation of C/EBPδ (Figure 7b), PPARγ (Figure 7c), C/EBPα (Figure 7d), and SREBP-1c (Figure 7e) and this effect was abrogated by co-incubation with zVAD.fmk. Comparable observations were made on protein level (Figure 5f).

These experiments demonstrate that TRAIL inhibits adipogenic differentiation by interfering with the expression of relevant transcription factors in a caspase-dependent manner.

**Discussion**

White adipose tissue is characterized by an enormous capability to either shrink or expand. This requires on the one hand tightly equilibrated metabolic processes regulating the volume of existing adipocytes, and on the other hand cellular processes regulating adipocyte number such as precursor cell proliferation, adipogenic differentiation, and cell death. In the present study we identified the death ligand TRAIL as a potent inhibitor of adipogenic differentiation. Via TRAIL-R2, TRAIL induced the activation of caspase-8 and -3, leading to reduced expression levels of the early core adipogenic transcription factors C/EBPα, C/EBPδ, and PPARγ, finally causing an inhibition of terminal differentiation and lipid accumulation.

TRAIL belongs to the TNF superfamily 2 and other family members were already described to have potent antiadipogenic properties. TNFα is the most prominent example, but also CD95L, TWEAK, and LIGHT inhibit adipogenesis. With this study we add TRAIL to the list of antiadipogenic factors. Interestingly though, TRAIL uses a distinct signaling route to mediate its effects on adipogenesis. While TNFα exerts its inhibitory function by NFκB activation, we did not detect any involvement of this pathway in mediating the effects of TRAIL. Alternatively, TNFα is able to block adipogenesis in murine 3T3-L1 cells via a β-catenin/TCF4 (TCF7L2)-dependent pathway. However, TRAIL does not seem to signal via this route as there was no TRAIL-dependent regulation of canonical Wnt target genes such as c-myc, cyclin D1, or PPARδ (data not shown) as shown for TNFα treatment. Also other TRAIL-activated non-canonical pathways such as ERK1/2, JNK, and p38 were excluded as players in the antiadipogenic action of TRAIL in human preadipocytes. A TRAIL-induced phosphorylation of ERK1/2 was observed at early time points of adipogenic differentiation. We have shown earlier that TRAIL induces proliferation in human preadipocytes in an ERK1/2-dependent manner. Therefore, we thought that TRAIL might push the cells into cell cycle and division and by this block the initiation of differentiation. However, cell numbers were not increased in TRAIL-treated cultures (data not shown) and inhibition of ERK1/2 activation with a small-molecule inhibitor of its upstream kinases MEK1/2 did not abrogate the effect of TRAIL, all in all arguing against an involvement of the ERK1/2 pathway in the antiadipogenic effect of TRAIL.
Instead, our data clearly indicate that TRAIL inhibits adipogenic differentiation via the canonical, caspase-dependent pathway. This is based on several pieces of evidence. First, TRAIL induced the cleavage and activation of caspase-8 and -3. Second, pharmacological inhibition of caspase activity, and third, genetic knockdown of caspase-8 expression completely abolished the antiadipogenic effect of TRAIL.

Caspases play an important role in differentiation processes in several cell types. For example, caspase-8 is involved in the differentiation of monocytes to macrophages and caspase-3 is crucial for the differentiation of mesenchymal stem cells along the osteogenic lineage. From our data we conclude that the adipogenic differentiation program per se does obviously not require the presence or activity of caspases since both the knockdown of caspase-8 as well as chemical inhibition of caspase activity had no effect on the differentiation rate and expression of adipocyte marker genes. They are, however, key players in mediating the antiadipogenic effect of TRAIL. In addition to the induction of cell death, TRAIL has been described as a modulator of differentiation processes in other studies. For example, TRAIL promotes the differentiation of intestinal cells and induces the maturation of both normal and leukemic myeloid precursor cells to monocytes. In contrast, TRAIL inhibits human erythropoiesis as well as the differentiation of osteoclasts. This underlines that TRAIL exerts its functions in a cell type-specific manner. The remaining question is how preadipocytes are able to survive an activation of caspase-3, which in apoptosis, together with the permeabilization of mitochondria, is often regarded the point of no return, where the destruction of a cell is inevitable. During the final steps of apoptosis, active caspase-3 is translocated to the nucleus to cleave nuclear substrates, which leads to typical cellular changes such as chromatin condensation and DNA fragmentation. In adipocytes, active caspase-3 was mainly detected in the cytoplasm after TRAIL treatment. This spatial distribution might provide one explanation why nuclear integrity is not altered and why there is no execution of cell death despite caspase activation following TRAIL.
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The consequence of TRAIL-induced caspase activation is a downregulation of the adipogenic transcription factors C/EBPα, C/EBPβ, and PPARγ on the mRNA and subsequently also on the protein level. Interestingly, the transcription factor C/EBPβ, which is induced very early in adipogenic differentiation,39 is not regulated by TRAIL on mRNA level, which leads us to conclude that TRAIL mediates its antiadipogenic effect downstream of C/EBPβ transcription. In line with our observations, TNFα treatment of 3T3-L1 cells under adipogenic conditions also leaves C/EBPβ transcription unaffected, while PPARγ and C/EBPα are robustly inhibited.29 PPARγ and C/EBPα mutually promote their expression40 and the transcriptional repression of these factors clearly suffices to inhibit adipogenesis. Both transcription factors are expressed on the mRNA level in the preadipocyte state before the onset of adipogenic differentiation (data not shown). Since caspases exert their functions by the cleavage of substrate proteins, we wondered whether these two transcription factors might be targeted and thereby inactivated. An ExPaSy peptide cutter analysis did not predict a cleavage of C/EBPα by caspases (data not shown). Likewise, no cleavage was predicted for C/EBPβ and C/EBPδ. In contrast, PPARγ is a known caspase substrate. TNFα was shown to induce PPARγ cleavage in murine adipocytes leading to its degradation.31,42 A comparable observation was made by our group. We demonstrated that cleaved PPARγ is retained in the cytoplasm instead of being translocated to the nucleus and therefore not able to induce the transcription of adipocyte-specific genes.15 However, in this study, PPARγ protein was first detectable by Western blot at 24 h after the induction of adipogenic differentiation and we were unable to detect a cleavage product following TRAIL treatment (data not shown). Using a proteasome inhibitor to prevent the degradation of a possible cleavage product yielded no meaningful insights because the co-treatment with TRAIL resulted in a rapid induction of cell death (data not shown). At this point we can thus not exclude a TRAIL-induced post-transcriptional modification of PPARγ by caspases.

In contrast to humans, mice possess only one death receptor with a functional intracellular domain, DR5, which corresponds to the human TRAIL-R2.1,2 Mice with a systemic knockout of DR5 have no overt adipose tissue phenotype. When maintained on a chow diet, their body weight and fat pad weights are comparable to those of wild-type littermates.43 On a diet high in saturated fat, cholesterol, and fructose (FCC), however, knockout animals gain significantly less weight and fat mass and are protected from the development of adipose tissue inflammation, insulin resistance, and hepatic steatosis.43 The absence of DR5 might lead to a reduction of apoptosis in adipose tissue and therefore also a decrease in macrophage infiltration and inflammation, which is regarded the initializing event in the pathophysiology of obesity-related co-morbidities.19 Alternatively, the phenotype could also be explained by a defect in macrophage function as macrophages derived from knockout animals displayed compromised TRAIL-induced chemotaxis as well as reduced cytokine production.42 Furthermore, DR5 might play a direct role in liver cell or adipocyte metabolism. For example, TRAIL was recently shown to induce insulin resistance in human adipocytes, inhibiting insulin-stimulated glucose uptake and lipogenesis.15 In contrast to this, the intraperitoneal injection of 10 μg TRAIL once per week resulted in reduced adiposity and improved insulin sensitivity and glucose tolerance in mice on a high-fat diet.44 Along the same line, mice lacking TRAIL expression displayed a more severely impaired glucose tolerance and increased systemic inflammation when on a high-fat diet. These controversial findings led us to conclude that the tissue-specific functions of TRAIL are still far from being understood, but will hopefully be dissected in the future by the use of tissue-specific DR5 knockout animals.

White adipose tissue is highly flexible with a unique capacity to change its volume by manifold. At least in mice both the expression of TRAIL and its receptor DR5 is upregulated in adipose tissue upon high-fat diet.15,42 Acute fasting in mice
leads to a downregulation of both ligand and receptor, and their expression is rapidly restored after re-feeding. This implies that the TRAIL/TRAIL-R system might have an important regulatory role in adipose tissue homeostasis. The finding that TRAIL inhibits adipogenesis and at the same time stimulates proliferation in preadipocytes suggests that it

![Graph](image)

**Figure 1.**

**a** Images of preadipocytes stained with Oil Red O on days 1, 4, 7, and 11 of differentiation for control and TRAIL-treated cells. TRAIL induces a significant increase in lipid accumulation compared to control. Each data point represents the mean ± SD of three independent experiments. **b** Graph showing cell number (×1000) for control and TRAIL-treated cells on days 0, 1, 4, 7, and 11. Control cells show a significant decrease in cell number compared to TRAIL-treated cells. **c** Graph showing the percentage of adherent cells on days 1, 4, 7, and 11. TRAIL-treated cells show a significant increase in adherent cells compared to control. **d** Graph showing specific apoptosis for control and TRAIL-treated cells on days 4 and 11. TRAIL-treated cells show a significant increase in apoptosis compared to control. **e** Graph showing the loss of MMP for control and TRAIL-treated cells on days 4 and 11. TRAIL-treated cells show a significant decrease in MMP compared to control. **f** Western blot analysis showing PARP and Lamin A/C expression in preadipocytes treated with TRAIL and CHX. TRAIL-induced apoptosis is confirmed by the increased cleavage of PARP and the decrease in Lamin A/C expression.
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The present study identified TRAIL as a potent antiadipogenic factor in human preadipocytes. TRAIL stimulates the cleavage of caspase-8 and -3, which results in the down-regulation of adipogenic transcription factors and decreases lipid accumulation. A decrease in adipogenesis might restrict the expansion of adipose tissue, but likewise increase the risk for ectopic lipid accumulation. TRAIL receptor agonists as well as human recombinant TRAIL are currently tested for their anticancer activity in phase II clinical studies. Further research is needed to clarify if targeting the TRAIL/TRAIL-R system is a useful strategy to improve or restore adipose tissue function in the context of obesity.

Materials and Methods

Recombinant human TRAIL was purchased from R&D Systems (#375-TEC; Wiesbaden-Nordenstadt, Germany). Fully human agonistic monovalent TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab) antibodies were kind gifts of Human Genome Sciences (Rockville, MD, USA). zVAD.fmK was purchased from Bachem (Bubendorf, Switzerland), SC-514 from Bio-Techne (Wiesbaden-Nordenstadt, Germany), PD98059 from Selleckchem (Houston, TX, USA), and cycloheximide from Sigma-Aldrich (Munich, Germany). Cell culture media and buffers were from Life Technologies (Darmstadt, Germany).

Figure 5

TRAIL induces negligible amounts of apoptosis during adipogenesis of SGBS cells. Human SGBS cells were treated with TRAIL (30 ng/ml) during the first 4 days of adipogenic differentiation. (a) Representative photomicrographs of cultures on days 1, 4, 7, and 11 of adipogenesis, magnification × 100. (b) The total number of cells and (c) the percentages of preadipocytes and adipocytes were determined by cell counting. Displayed are the means and S.E.M. of three independent experiments. (d) Knockdown of caspase-8 was controlled by western blot. One representative out of four experiments performed is presented. (e) Transduced SGBS cells were treated with TRAIL (10 ng/ml) and the rate of adipogenic differentiation was determined by cell counting on day 10 of differentiation. Displayed is the mean and S.E.M. of three independent experiments. One-way ANOVA and Turkey's multiple comparison were used to test for statistical significance in (b, c, and e). *P < 0.05; **P < 0.01; ***P < 0.001, vehicle versus TRAIL and/or zVAD.

Figure 6

The antiadipogenic effect of TRAIL is mediated by caspases. (a-c) SGBS cells were treated with TRAIL (30 ng/ml) in the absence or presence of the pan-caspase inhibitor zVAD.fmK (20 μM). (a) Inhibition of caspases by zVAD.fmK was confirmed by western blot after 3 h of treatment. One representative out of three experiments performed is presented. The positions of the molecular weight markers (kDa) are indicated. (b) The rate of adipogenic differentiation was determined by cell counting on day 10 of differentiation. Displayed are the means and S.E.M. of three independent experiments. (c) RNA was isolated and adipocyte marker gene expression (PPARγ, Glut-4, adiponectin) was determined by qPCR on day 10 of differentiation. The mRNA levels were normalized to the gene HPRT. Displayed are the means and S.E.M. of three independent experiments. (d and e) SGBS cells were transduced with lentiviruses expressing either a non-targeting shRNA sequence (hyper random sequence, HRS) or shRNA targeting casp-8.2 (C8.1 and C8.2) to generate a stable knockdown. (d) Knockdown of caspase-8 was confirmed by western blot after 3 h of treatment. One representative out of three experiments performed is presented. (e) Transduced SGBS cells were treated with TRAIL (10 ng/ml) and the rate of adipogenic differentiation was determined by cell counting on day 10 of differentiation. Displayed is the mean and S.E.M. of three independent experiments. One-way ANOVA and Turkey's multiple comparison were used to test for statistical significance in (b, c, and e). *P < 0.05; **P < 0.01; ***P < 0.001, vehicle versus TRAIL and/or zVAD.
Subjects and human primary stromal-vascular cell isolation. Primary human stromal-vascular cells were isolated from subcutaneous white adipose tissue by collagenase (Sigma-Aldrich, Munich, Germany) digestion from five female subjects undergoing plastic surgery and cultured as described. The mean age was $39.2 \pm 9.6$ years; the mean BMI was $30.0 \pm 3.0$ kg/m$^2$. All procedures were performed according to the Declaration of Helsinki guidelines and authorized by the ethics committee of Ulm University. Written informed consent was obtained from all subjects in advance.

Cell culture. Human SGBS cells were used as a model system for adipogenesis. To induce adipogenic differentiation in SGBS and primary human stromal-vascular cells, subconfluent cell cultures were washed with PBS and adipogenic basal medium (serum-free DMEM-F12 (1:1) with $33 \mu$M biotin, $17 \mu$M pantothenate, $20$ nM human insulin, $100$ nM cortisol, $200$ nM triiodothyronine, and $10$ μg/ml transferrin) supplemented with $2$ μM rosiglitazone, $25$ nM dexamethasone, and $250$ μM isobutylmethylxanthine was added. TRAIL was added to the cultures for the first 4 days of adipogenesis. After 4 days, the medium was changed to adipogenic basal medium without TRAIL. Analyses were performed 10 days after the induction of adipogenesis. The differentiation rate was determined by counting the number of lipid-laden, differentiated adipocytes (defined by five clearly visible lipid droplets) and undifferentiated cells. Three microscopic fields were counted per well using a net micrometer.

Figure 7 TRAIL reduces the expression of adipogenic transcription factors. SGBS cells were treated with TRAIL (30 ng/ml) in the absence or presence of the pan-caspase inhibitor zVAD.fmk (20 μM) for the first 4 days of adipogenic differentiation. RNA was isolated after 48, 72, 120, 168, and 240 h and qPCR analysis of the adipogenic transcription factors C/EBPβ (a), C/EBPδ (b), PPARγ (c), C/EBPα (d), and SREBP1C (e) was performed. The mRNA levels were normalized to the gene HPRT. Displayed are the means and S.E.M. of three independent experiments. (f) Protein was isolated after 7 and 10 days and the protein expression of C/EBPβ, C/EBPδ, PPARγ, C/EBPα, and SREBP1C was analyzed by western blot. α-Tubulin was used as a loading control. The positions of the molecular weight markers (kDa) are indicated. One representative out of three experiments performed is presented. Two-way ANOVA and Turkey’s multiple comparison were used to test for statistical significance in (a–e). *$P<0.05$; **$P<0.01$; ***$P<0.001$, vehicle versus TRAIL.
Caspase activity assay. Caspase activity was quantified using CellEvent Caspase-3/7 Green Detection Reagent (Life Technologies). After TRAIL treatment the cells were trypsinized and stained for 30 min at 37 °C with the detection reagent at a final concentration of 2 μM. Cell analysis was performed using a FACScanBul flow cytometer (BD, Franklin Lakes, USA). For each treatment triplicate wells were prepared.

Apoptosis assays. Apoptosis was determined by fluorescence-activated cell-sorting (FACS)Bul; Becton Dickinson, Heidelberg, Germany) analysis of DNA fragmentation of propidium iodide-stained nuclei as previously described.17 For each treatment triplicate wells were prepared. Specific apoptosis was calculated using the following formula: (observed apoptosis − spontaneous apoptosis) × (100/ (100 − spontaneous apoptosis)).

The mitochondrial membrane potential was determined as previously described.17 For each treatment triplicate wells were prepared. Briefly, CMXRoB (Molecular Probes, Karlsruhe, Germany) was added to the cultures at a final concentration of 35 nM. Cells were incubated for 30 min at 37 °C in the presence of the dye and then immediately analyzed by flow cytometry.

Statistical analysis. All statistical analyses were performed using GraphPad Prism software version 6.01 (La Jolla, CA, USA).

Conflict of Interest. The authors declare no conflict of interest.

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**Supplementary Information** accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)