Tumour necrosis factor-α inhibits adipogenesis via a β-catenin/ TCF4(TCF7L2)-dependent pathway

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Abstract

Tumour necrosis factor-α (TNF-α), a proinflammatory cytokine, is a potent negative regulator of adipocyte differentiation. However, the mechanism of TNF-α-mediated antiadipogenesis remains incompletely understood. In this study, we first confirm that TNF-α inhibits adipogenesis of 3T3-L1 preadipocytes by preventing the early induction of the adipogenic transcription factors peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein-α (C/EBPα). This suppression coincides with enhanced expression of several reported mediators of antiadipogenesis that are also targets of the Wnt/β-catenin/T-cell factor 4 (TCF4) pathway. Indeed, we found that TNF-α enhanced TCF4-dependent transcriptional activity during early antiadipogenesis, and promoted the stabilisation of β-catenin throughout antiadipogenesis. We analysed the effect of TNF-α on adipogenesis in 3T3-L1 cells in which β-catenin/TCF signalling was impaired, either via stable knockdown of β-catenin, or by overexpression of dominant-negative TCF4 (dnTCF4). The knockdown of β-catenin enhanced the adipogenic potential of 3T3-L1 preadipocytes and attenuated TNF-α-induced antiadipogenesis. However, β-catenin knockdown also promoted TNF-α-induced apoptosis in these cells. In contrast, overexpression of dnTCF4 prevented TNF-α-induced antiadipogenesis but showed no apparent effect on cell survival. Finally, we show that TNF-α-induced antiadipogenesis and stabilisation of β-catenin requires a functional death domain of TNF-α receptor 1 (TNFR1). Taken together these data suggest that TNFR1-mediated death domain signals can inhibit adipogenesis via a β-catenin/ TCF4-dependent pathway.

Keywords

adipocyte differentiation; adipogenesis; Wnt signalling; TNF-alpha; β-catenin; TCF4; TCF7L2; TNFR1

The formation of new adipocytes from precursor cells by adipogenesis is important for normal adipose tissue function. Indeed, impaired adipogenesis may contribute to the pathogenesis of obesity-associated conditions including insulin resistance, hyperlipidaemia and type II diabetes. Adipogenesis involves the activation of a cascade of transcription

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factors, which coordinate the expression of genes responsible for adipocyte function.[1] Adipogenic stimuli induce the rapid expression of the transcription factors CCAAT/ enhancer-binding protein-β (C/EBPβ) and C/EBPδ, which stimulate a transient stage of mitotic clonal expansion during early adipogenesis.[1] C/EBPβ and C/EBPδ also promote the expression of the master adipogenic transcription factors C/EBPα and peroxisome proliferator-activated receptor-gamma (PPARγ). Together these act cooperatively to regulate the expression of genes associated with terminal differentiation.[1] Local and endocrine factors may regulate adipogenesis by modulating the activity of these transcriptional events.[2]

Negative regulators of adipogenesis include canonical Wnt signalling[3] and inflammatory cytokines such as tumour necrosis factor-α (TNF-α).[4] Canonical Wnt signalling inhibits adipogenesis by stabilising β-catenin, a multifunctional protein involved in cell adhesion and transcriptional regulation. In the absence of Wnt stimulation, cytoplasmic β-catenin is phosphorylated by a multiprotein 'destruction complex', which targets β-catenin for ubiquitination and proteasomal degradation.[5] Wnt ligands promote the dissociation of this destruction complex and thereby prevent β-catenin degradation.[5] Consequently, cytoplasmic β-catenin accumulates and translocates into the nucleus where it co-activates the T-cell factor (TCF)/lymphoid enhancer factor family of transcription factors to induce Wnt/β-catenin target gene expression.[5] Both dividing and confluent 3T3-L1 preadipocytes express Wnt-10b, which activates canonical Wnt signalling, stabilises β-catenin and inhibits adipogenesis. Physiological induction of adipogenesis coincides with the downregulation of both Wnt-10b and β-catenin.[3,6] Conversely, forced stabilisation of β-catenin inhibits adipogenesis.[3] Hence, signalling via β-catenin/TCF may be important in the physiological regulation of adipocyte differentiation.

In contrast, inhibition of adipogenesis by TNF-α may be more relevant to pathological conditions. Adipogenesis is often impaired in disorders associated with elevated production of TNF-α, such as in cachexia, sepsis and in obesity-related insulin resistance.[7] The latter is based on the hypo thesis that restriction of adipose tissue expandability may contribute to obesity-related hyperlipidaemia and subsequently leading to lipotoxicity-induced insulin resistance.[8] The antiadipogenic actions of TNF-α have been primarily attributed to signals transduced by TNF-α receptor-1 (TNFR1),[4,9] through which TNF-α stimulates numerous signalling pathways. These include the activation of sphingomyelinases, caspases, JNK, Erk1/2 and nuclear factor-kappa B (NF-κB). Each of these have been implicated in the negative regulation of adipogenesis.[9] Although it has been suggested that activation of NF-κB is necessary for TNF-α-induced antiadipogenesis,[10,11] this conflicts with the recent finding that NF-κB activity increases during adipogenesis.[12] Hence, the nature of the final antiadipogenic signalling pathway(s) induced by TNFR1 remains incompletely understood.

Here, we confirm that TNF-α inhibits adipogenesis by preventing the early induction of PPARγ and C/EBPα. However, this effect coincides with enhanced expression of several antiadipogenic genes that are also targets of the Wnt/β-catenin/T-cell factor 4 (TCF4) pathway. Therefore, we investigated whether TNF-α inhibits adipogenesis by activating the β-catenin/TCF4 pathway. Our results show that TNF-α enhances the transient β-catenin/ TCF4 transcriptional activity during early antiadipogenesis. Blocking this pathway via stable

knockdown of β-catenin or overexpression of dominant-negative TCF4 (dnTCF4) attenuates TNF-α-induced antiadipogenesis. Therefore, we propose that TNF-α inhibits adipogenesis via a β-catenin/TCF4 dependent pathway.

Results

Short-term exposure to TNF-α is sufficient to inhibit adipogenesis in 3T3-L1 cells

To identify the earliest adipogenic events that are impaired by TNF-α we first investigated the impact of limiting TNF-α exposure to the first 48 h of 3T3-L1 adipogenesis. Figure 1a shows that TNF-α mediates the same antiadipogenic effects whether it is present for the first 48 h of adipogenesis or throughout the 8-day adipogenic programme. The 48 h TNF-α treatment significantly prevented the expression of aP2 that occurred during normal adipogenesis (Figure 1b). This is consistent with the previously reported effects on F442A preadipocytes[13] and suggests that this TNF-α action is not cell-type specific and antiadipogenic effects occur early during the induction phase of the adipogenesis.

To investigate further the temporal characteristics of TNF-α-induced antiadipogenesis, we analysed expression of several key adipogenic transcription factors. In control cells, adipogenic induction significantly increased the expression of PPARγ2 from 8 h post-induction onwards, and increased C/EBPα expression was detected within 4 h post-induction (Figure 1c and d). Thus the increased expression of these key adipogenic transcription factors occurs earlier than has been previously reported.[14,15] TNF-α treatment completely prevented the induction of both PPARγ2 and C/EBPα (Figure 1c and d). Additionally, TNF-α suppressed the expression of C/EBPα to below pre-induction levels, reducing it by approximately 60% at 8 h post-MDI (Figure 1d). In contrast, TNF-α did not alter the expression of C/EBPβ, an inducer of C/EBPα and PPARγ (Figure 1e). These data show that TNF-α mediates its antiadipogenic effects early in the adipogenic programme.

TNF-α enhances the transient expression of antiadipogenic mediators during the early stages of antiadipogenesis

To investigate how TNF-α suppresses C/EBPα and PPARγ2 expression, we analysed the expression of transcription factors known to inhibit these adipogenic genes. GATA2 and GATA3 suppress PPARγ by directly binding to its promoter, and forced expression of GATA2 or GATA3 inhibits adipogenesis.[16] In control cells, both GATA2 and GATA3 mRNAs were downregulated within 2–4 h post-induction. However, neither of these mRNAs showed altered expression in TNF-α-treated cells (Figure 2a and b). Similarly, TNF-α stimulates the sustained expression of c-myc during antiadipogenesis in TA1 preadipocytes[17] and forced expression of c-myc prevents adipogenesis by inhibiting the expression of C/EBPα.[18] Additionally, both cyclin D1 and PPARδ inhibit adipogenesis by suppressing PPARγ.[19,20] Figures 2c–e show that the mRNA expression of c-myc, cyclin D1 and PPARδ was significantly upregulated following adipogenic induction, and this expression was further enhanced by TNF-α treatment. At later time-points (4–8 days), only cyclin D1 expression remained elevated in TNF-α-treated cells (Figure 2d).

TNF-α activates β-catenin/TCF signalling during antiadipogenesis

Intriguingly, c-myc, cyclin D1 and PPARδ are target genes of the canonical Wnt signalling pathway, which itself has been shown to inhibit adipogenesis.[21-23] We next investigated Wnt10b mRNA expression. Under control conditions, there was a transient peak in Wnt10b mRNA levels, which subsequently dropped to below preadipocyte levels (Figure 3a). In contrast, Wnt10b levels during TNF-α-induced antiadipogenesis were neither elevated nor maintained in the first 48 h of the programme. Instead, Wnt10b transcript levels were significantly suppressed by TNF-α treatment and only began to return to normal after TNF-α was removed.

We next investigated whether the TNF-α-induced upregulation of c-myc, cyclin D1 and PPARδ represented a more specific effect of TNF-α on β-catenin/TCF transcriptional activity using a TCF-dependent luciferase reporter, TOP-FLASH. TNF-α enhanced TOPFLASH-dependent luciferase activity at 12 h post-induction of adipogenesis (Figure 3b) but not at 48 h post-induction (Figure 3c). This is consistent with the early but transient enhancement of wnt-target genes during antiadipogenesis (Figure 2c–e). To investigate whether TNF-α mediates its effects on TCF-dependent gene expression via stabilisation of β-catenin, cytosolic protein levels were examined. As shown in Figure 3d, TNF-α did not affect the levels of cytosolic β-catenin during the first 48 h post-induction. However, at later time points (>48 h), β-catenin protein levels were maintained in TNF-α treated cells (Figure 3e and f). In contrast, TNF-α did not alter the expression of β-catenin mRNA during antiadipogenesis (Figure 3g). However, the constitutive expression of a stable β-catenin mutant (β-catenin S45A)[24] also inhibited adipogenesis (Figure 3h). This suggests that TNF-α-induced stabilisation of β-catenin may contribute to the antiadipogenic actions of TNF-α.

β-catenin knockdown enhances 3T3-L1 adipogenesis and attenuates TNF-α-induced antiadipogenesis

To address the role of β-catenin in TNF-α-induced antiadipogenesis, we generated a 3T3-L1 cell line in which expression of β-catenin is stably knocked down by ectopic expression of a short hairpin RNA (shRNA) specific to β-catenin. The expression of β-catenin mRNA and protein was significantly reduced in these cells (Figure 4a and b). The adipogenic potential of this cell line appeared similar to control preadipocytes, with both differentiating completely in response to full induction cocktail and with no morphological differences apparent at day 8 (Figure 4c). However, when subjected to submaximal induction, the β-catenin knockdown preadipocytes exhibited enhanced lipogenesis (Figure 4c) and consistently expressed higher levels of PPARγ2 and C/EBPα (Figure 4d and e). Surprisingly, β-catenin knockdown cells also expressed more PPARγ2 mRNA when induced with full MDI (Figure 4d). These results indicate that knockdown of β-catenin enhances adipogenesis in 3T3-L1 preadipocytes. Furthermore, they suggest that β-catenin may normally limit PPARγ2 and C/EBPα expression in mature 3T3-L1 adipocytes.

The requirement for β-catenin in TNF-α-induced antiadipogenesis was investigated by inducing control and β-catenin knockdown cells to differentiate with MDI in the presence of a range of concentrations of TNF-α. Figure 4f shows that 2–5 ng/ml TNF-α completely prevented lipid accumulation in both control and β-catenin knockdown cells. However,

1ng/ml TNF-α was less effective at inhibiting lipid accumulation in β-catenin knockdown cells. Analysis of PPARγ2 and C/EBPα mRNA expression confirmed that TNF-α inhibited their expression in a concentration-dependent manner (Figure 4g and h). However, in β-catenin knockdown cells, the concentration-dependent expression of C/EBPα (Figure 4h) was right-shifted. This indicates that TNF-α may regulate C/EBPα expression in a β-catenin-dependent manner.

β-catenin knockdown promotes apoptosis during TNF-α-induced antiadipogenesis

A striking observation made during our experiments was that β-catenin knockdown preadipocytes were more sensitive to TNF-α-induced cell death during adipogenic treatment. This observation was confirmed by subsequent assessment of adherent and viable cell numbers (at 24 h). In control cells, TNF-α had no significant effect on cell viability during antiadipogenesis (Figure 5a and b). In contrast, in β-catenin knockdown cells, TNF-α significantly reduced the number of viable cells (Figure 5a and b), suggesting that TNF-α-induced cytotoxicity may be enhanced in the absence of β-catenin. Consistent with this, TNF-α-induced DNA fragmentation was markedly enhanced in the β-catenin knockdown cells (Figure 5c and d).

dnTCF4 reverses TNF-α-induced antiadipogenesis but not TNF-α-induced stabilisation of β-catenin

As β-catenin has numerous functions, including cellular adhesion and transcriptional regulation, we took an alternative approach to address the specific requirement of β-catenin/TCF signalling in TNF-α-induced antiadipogenesis. Expression of a dominant negative mutant of TCF4 (dnTCF4) blocks β-catenin/TCF4-dependent transcription.[25] Figure 6a shows that this can be reproduced in 3T3-L1 adipogenesis. In response to adipogenic induction with MDI, dnTCF4 cells appeared to differentiate to the same extent as control cells, as assessed by lipid accumulation and expression of adipogenic markers at day 8 post-induction (Figure 6b and c). Interestingly, TNF-α treatment markedly suppressed lipid accumulation in control cells but had little effect on lipid accumulation in dnTCF4-expressing cells (Figure 6b). Furthermore, TNF-α-induced suppression of aP2, PPARγ2 and C/EBPα expression was completely prevented in dnTCF4-expressing cells (Figure 6c).

As dnTCF4-expressing cells were resistant to TNF-α-induced antiadipogenesis, we tested whether the TNF-α-induced stabilisation of β-catenin seen at later stages of antiadipogenesis (Figure 2d and e) was also prevented in this model. Surprisingly, cytosolic β-catenin protein levels were also maintained in dnTCF4-expressing cells treated with TNF-α (Figure 6d and e). These results suggest that the transcriptional activity of β-catenin via TCF4 is required for TNF-α-induced antiadipogenesis. Furthermore, they show that the stabilisation of β-catenin is associated with TNF-α treatment, rather than being a consequence of antiadipogenesis *per se*.

A functional TNFR1-death domain is required for antiadipogenesis and β-catenin stabilisation by TNF-α

To elucidate which signals mediated by TNFR1 are important in inhibiting adipogenesis and stabilising β-catenin, we investigated the requirement of specific domain(s) of TNFR1.

Initial studies using TNFR1$^{-/-}$ R2$^{-/-}$ preadipocyte cell lines ectopically expressing deletion or point mutants of TNFR1 (Figure 7a) showed that the antiadipogenic actions of TNFR1 mutants, particularly TNFR1- CT and TNFR1-L351A, were accompanied by TNF-α-induced cell death (data not shown). The varying levels of stable expression obtained also correlated inversely with cytotoxic functionality. However, these studies identified loss-of-function TNFR1 mutants; namely TNFR1- DD CT, TNFR1-KFRA and, to a lesser extent, TNFR1- JM. To test whether these mutants had dominant-negative properties, we established wild-type 3T3-L1 preadipocyte cell lines ectopically expressing these TNFR1 mutants. Each cell line was then tested for sensitivity to TNF-α-induced antiadipogenesis (Figure 7b). Although cells expressing TNFR1- CT or TNFR1-L351A were comparable to empty vector cells, those expressing TNFR1- DD CT or TNFR1-KFRA (and to a lesser extent TNFR1- JM), were more resistant to the actions of TNF-α (Figure 7b). Again lipid accumulation correlated with the expression of adipogenic genes, PPARγ C/EBPα, aP2 and Glut4 (data not shown). These data confirm that a functional TNFR1-death domain is required for mediating the antiadipogenic actions of TNF-α in 3T3-L1 preadipocytes.

We examined whether a functional TNFR1-DD is also required for TNF-α-induced stabilisation of β-catenin. Figures 7c and d show that expression of the dominant negative mutants; TNFR1- DD CT, TNFR1- JM or TNFR1-KFRA prevented TNF-α-induced stabilisation of β-catenin. This was not observed in cells expressing TNFR1- CT or TNFR1-L351A. These data suggest that signals stimulated by a functional TNFR1-DD are necessary for both TNF-α-induced antiadipogenesis and stabilisation of β-catenin.

Discussion

Both TNF-α and canonical Wnt signalling inhibit adipogenesis early in differentiation and suppress the induction of PPARγ and C/EBPα. Despite these functional similarities, two very different signalling pathways have been reported to mediate the antiadipogenic signals downstream of TNF-α and Wnt10b receptors. Here we provide evidence to suggest that TNF-α and Wnt signalling pathways may converge to inhibit adipocyte development at the level of TCF4-dependent gene transcription. We demonstrate that TNF-α utilises components of the canonical Wnt signalling pathway during antiadipogenesis. Additionally, we show that short-term TNF-α treatment early in adipogenesis (<48 h) results in subsequent maintenance of cytosolic stable β-catenin (>4 days). Therefore, whereas β-catenin/TCF4 activity is important in inhibiting adipogenesis, β-catenin may also play a role in maintaining TNFα-induced antiadipogenesis.

Transcriptional events and canonical Wnt signalling during early adipogenesis

3T3-L1 preadipocytes are arguably the most well-characterised cell lines with respect to the transcriptional regulation of adipogenesis. In these cells, events occurring within the first 24 h of adipogenesis are linked to regulating synchronised cell cycle and include the transient expression of oncogenic genes such as cyclin D1 and c-myc. We have observed that many of these genes are also TCF target genes and this is entirely consistent with our observation that Wnt10b expression increases transiently, shortly after induction. In contrast, MacDougald and others[26,27] originally reported that Wnt10b mRNA is immediately

suppressed by adipogenic stimulation in the same cell types. This disparity may be owing to differences in sampling frequency. Nevertheless, given the timing and transient nature of this event, just prior to the onset of transient proliferative signals and clonal expansion, it is tempting to speculate that activation of the canonical Wnt-10b pathway may be the trigger for entry into the clonal expansion phase of adipogenesis. Subsequent inhibition of this pathway may then allow lipogenesis and terminal differentiation to proceed.

Mechanistically, canonical Wnt signalling acts by stabilising cytosolic β-catenin, which then translocates to the nucleus where it co-activates LEF/TCF transcription factors. An intriguing observation made in our study is that Wnt10b levels and TCF transcriptional activity are transiently upregulated within the first 24 h of induction, but we were unable to detect significant changes in cytosolic β-catenin levels within this period. It is only after day 2 of the differentiation programme that cytosolic β-catenin levels appear to fall. However, other studies have detected such increases in nuclear β-catenin levels at similar times in both murine and human preadipocytes.[6,28] Whether canonical Wnt signalling is a prerequisite for adipogenesis of human preadipocytes also seems a likely possibility as components of the canonical Wnt signalling pathway have recently been reported to be coordinately regulated during this process.[28] Nonetheless, this remains to be formally established, as does the putative role of canonical Wnt signalling in adipose tissue development *in vivo*.

Mechanisms of TNF-α-induced antiadipogenesis

This study addresses the temporal characteristics of the antiadipogenic signals induced by TNF-α. To date, most investigations into TNF-α-induced antiadipogenesis have studied the effects of chronic TNF-α treatment by maintaining TNF-α in adipogenic medium throughout the differentiation protocol.[9,27] However, by limiting TNFα treatment to the induction phase of adipogenesis (first 48 h), we show that TNF-α targets the early events of 3T3L1 differentiation and that this is sufficient to completely inhibit adipogenesis. At the transcriptional level, TNF-α treatment has little effect on GATA2/3, C/EBPβ (Figures 1 and 2) or C/EBPδ (unpublished data) mRNA but completely prevents the induction of PPARγ2 and C/EBPα mRNA. In addition, basal C/EBPα levels are significantly suppressed. As PPARγ and C/EBPα promote the expression of one another[29] (Figure 8), this early suppression of C/EBPα expression may be sufficient to inhibit adipogenesis in response to TNF-α treatment.

At earlier time points (<24 h), TNF-α induced antiadipogenesis is accompanied by an enhanced induction of the oncogenic genes c-myc, cyclin D1 and PPARδ. As forced expression of c-myc prevents adipogenesis by inhibiting the expression of C/EBPα,[18] c-myc is a good candidate for mediating TNF-α-induced antiadipogenesis via the suppression of basal C/EBPα expression in our system. Additionally, the enhanced expression of both cyclin D1 and PPARδ may themselves contribute to inhibiting adipogenesis by suppressing any basal PPARγ activity in preadipocytes.[19,20]

In line with the observation that c-myc, cyclin D1 and PPARδ are also β-catenin/TCF4 target genes, we further demonstrate that TNF-α-induced signals can specifically enhance β-catenin/TCF4 activity and may thereby alter events that occur upstream of C/EBPα and PPARγ expression (Figure 8). Furthermore, we show that knockdown of β-catenin or

overexpression of dnTCF4 attenuates TNF-α-induced antiadipogenesis. This indicates that signalling via β-catenin/TCF4 is important for the antiadipogenic effects of TNF-α. Interestingly, C/EBPα may be reciprocally linked to β-catenin/TCF activity as C/EBPα levels are elevated when β-catenin is knocked down (Figure 4e). This molecular connection warrants further investigation.

To elucidate further the mechanism underlying TNF-α-induced activation of β-catenin/TCF activity, we measured Wnt10b mRNA levels during TNF-α-induced antiadipogenesis. Surprisingly, rather than being maintained or upregulated, Wnt10b levels were dramatically suppressed by TNF-α treatment and no transient increase was detected. However, Wnt10b expression did return to basal levels but only at later stages of antiadipogenesis. Taken together, these findings indicate that TNF-α signalling in the presence of adipogenic induction cocktail may bypass Wnt10b mRNA expression to activate β-catenin/TCF target gene expression and suppress C/EBPα expression.

Regulation of apoptosis during antiadipogenesis

An established aspect of TNF-α biology is its ability to induce cell death. This has been suggested to be a mechanism by which TNF-α prevents both adipogenesis *in vitro* and increased adipose tissue mass *in vivo*. Here we show that the low concentrations of TNF-α used in our study completely inhibit adipogenesis without inducing apoptosis. This is consistent with at least one other independent study.[10] Nonetheless, it appears that β-catenin may play an important role in determining cell survival during TNF-α-induced antiadipogenesis (Figure 5). This is reminiscent of the antiapoptotic effects of Wnt signalling via β-catenin/TCF4-dependent transcription.[30] However, as we did not observe increased TNF-α-induced apoptosis in dnTCF4-expressing cells, we cannot rule out the possibility that β-catenin promotes cell survival independently of TCF4, for example through cell adhesion-dependent signals.[31]

Of note is that TCF4 is also called transcription factor 7 like-2 (TCF7L2), and it has been linked to maintenance of stem cell phenotypes[32] and preventing differentiation or apoptosis. Intriguingly, TCF7L2 expression is significantly decreased in adipose tissue of obese type 2 diabetic subjects compared with obese normoglycemic individuals.[33] Furthermore, recent studies have identified a strong association between at least 2 SNPS in the TCF7L2 gene and type II diabetes.[34] However, these risk-conferring genotypes are not associated with insulin resistance but rather with impaired β-cell function, development and possibly survival.[33,35]

Maintenance of β-catenin levels during antiadipogenesis

The stability of β-catenin beyond 48 h in differentiating adipocytes has itself been the subject of recent investigations. Reports from Farmer and colleagues[6,36,37] suggest that proteasomal degradation of β-catenin during adipogenesis is dependent on PPARγ activation. Therefore, TNFα-induced stabilisation of β-catenin may involve suppression of PPARγ activity. Indeed, we have found that the PPARγ ligand rosiglitazone can partially reverse TNF-α-induced stabilisation of β-catenin (unpublished data). However, this mechanism is not consistent with the observation that β-catenin levels are maintained in cells that can differentiate in the presence of TNF-α (i.e. in cells expressing dnTCF4). In our

study we show that PPARγ is expressed and is active, since appreciable levels of aP2 (a PPARγ-target gene) are detected. An alternative mechanism, supported by our data, is that maintenance of β-catenin levels in TNF-α-treated cells beyond the induction period is mediated by the return of Wnt10b expression to preadipocyte levels (Figure 3a). Interestingly, this has also recently been observed in preadipocytes treated with TNF-α throughout the adipogenic programme.[27]

Cross-talk between Wnt and TNF-α signals during antiadipogenesis

TNF-α-induced antiadipogenesis has been attributed to signals primarily transduced by TNFR1.[4,9] In this study, we show that a functional TNFR1-DD is required and sufficient for both antiadipogenesis and subsequent maintenance of β-catenin by soluble TNF-α. This reduces the potential candidate signals responsible for mediating these effects to include the serine kinases ERK1/2, JNK, NFκB, p38 and aSMase, cPLA2, and caspase activation (Figure 7a). Although this is consistent with reports suggesting that NFκB is required for TNF-α-induced antiadipogenesis,[10,11] it is also noteworthy that several of these signals have themselves been implicated in regulating β-catenin/TCF activity.[38-40] Intriguingly, the activation of these signals by TNF-α is also acute and transient.[10,41] Which of these regulate β-catenin/TCF activity during TNF-α-induced antiadipogenesis is currently under investigation.

In conclusion, our findings suggest that early signals induced by TNF-α via the death domain of TNFR1 are required to mediate downstream effects on β-catenin/TCF4 activity and that these are necessary for TNF-α-induced antiadipogenesis (Figure 8). This novel pathway provides a potential mechanism by which TNF-α can impact on cell fate determination. Indeed, it may be involved in mediating the impaired adipogenesis and adipose tissue expandability that is associated with obesity-related hyperlipidaemia and insulin resistance. Furthermore, this finding has important implications for our understanding of the growth factor-like aspects of TNF-α biology and may provide a new molecular link between low-grade inflammation (as seen in obesity) and the associated increased risk of obesity-related cancer. Future investigations should explore this possibility.

Materials and Methods

Materials

Tissue culture media, insulin, IBMX, dexamethasone, oil red O and puromycin were from Sigma (St Louis, MO, USA). Tissue culture sera were from HyClone (Logan, UT, USA). Murine recombinant TNF-α was from R&D Systems (Minneapolis, MN, USA). TNF-α concentrations are presented in ng/ml, where 1 ng/ml corresponds to approximately 85 pM TNF-α. The anti-β-catenin antibody was purchased from Transduction Laboratories (Oxford, UK). The anti-PPARγ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). These commercially available antibodies were used according to the manufacturer's instructions. The anti-p85 antibody was kindly provided by K. Siddle (Cambridge, UK) and was used at a 1: 1000 dilution in phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA). The anti-aP2 antibody was kindly provided by DA Bernlohr (University of Minnesota, MN, USA) and was used at a 1: 5000 dilution in TBST

+ 1% BSA. All horseradish peroxidase-conjugated secondary antibodies were purchased from DakoCytomation, (Ely, UK) and used at a 1: 10 000 dilution in TBST + 1% BSA.

Retroviral constructs

DnTCF4 lacks the first 31 amino acids (i.e. the β-catenin-binding domain) of TCF4.[24] This was excised from pcDNA3 with Kpn1/Xba1 digest, Klenow filled-in and blunt-end-ligated into the SnaB1 site of pBabe-puro. Correct orientation was confirmed by at least three different diagnostic digests. Generation of pBabe-mTNFR1wt (gb: M60468), pBabe-mTNFR1 DD CT (lacking the last 101 amino acids) and pBabe-mTNFR1 JM have been reported previously.[9] In addition to these constructs, we generated pBabe-TNFR1 CT, pBabe-mTNFR1-L351A and pBabe-mTNFR1-KFRA. TNFR1 CT was created by mutating L412 to a stop codon and expresses mTNFR1 lacking the last 14 amino acids that are required for induction of iNOS.[42] TNFR1-L351A is a full-length TNFR1 harbouring an inactivating point mutation that is analogous to the inactivating LPR mutation of Fas death domain.[43] Similarly, TNFR1-KFRA is a full-length TNFR1 with three point mutations K343A F345A and R347A, which can also inactivate death domain signals.[44] The cDNA inserts were created by site-directed mutagenesis of pCRII(+)-mTNFR1wt using QuickChange (Strategene, Cedar, Creek, TX, USA) and these were confirmed by direct sequencing. Receptor mutants were then released with sequential *Nae1* and *Eco*RI digests and directionally ligated into *SnaB1-EcoRI*-digested pBabe-Puro.

Stable knockdown of β-catenin was achieved by expression of a shRNA from the pSiren-RetroQ vector (Clontech, Mountain View, CA, USA). Target sequences for knockdown of β-catenin were identified using the Dharmacon siDesign center. Custom oligonucleotides (sequences available on request) were designed to incorporate these identified sequences into an shRNA expression sequence and cloned into pSiren-RetroQ according to the manufacturer's instructions.

Cell culture and generation of stable preadipocyte cell lines

3T3-L1 cells were cultured, differentiated into adipocytes and stained for Oil red O as described previously.[45] Cells were analysed by phase contrast microscopy using a Nikon Eclipse TE300 inverted microscope.

3T3-L1 and TNFR1 $^{-/-}$R2$^{-/-}$ cell lines that stably overexpress dnTCF4, TNFR mutants or control vectors were generated with the pBabe-Puro retroviral vector system as described.[45] 3T3-L1 cells with shRNA-mediated knockdown of β-catenin (or controls) were generated with the pSiren-RetroQ retroviral vector system, as done for the pBabe-Puro system. All retrovirally transfected 3T3-L1 cell lines were kept in puromycin-containing medium throughout culture and differentiation procedures.

TCF4 reporter assay

To assay for activation of β-catenin/TCF target genes, 3T3-L1 cells were seeded in 24-well plates 1 day before being transfected with 1 μg of Topflash TCF reporter construct (Upstate, Charlottesville, VA, USA). Transfection was done using Fugene 6 reagent (Roche Applied Science, Burgess Hill, UK) according to the manufacturer's protocol. Three days post-

transfection, cells were induced to differentiate in the absence or presence of TNF-α. Luciferase activity was assayed using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Values were normalized to the activity of co-transfected phRL-CMV-Luc constitutive Renilla luciferase reporter vector (Promega).

Western blotting

Before protein analysis by Western blotting, medium was removed and cell monolayers were washed with ice-cold Dulbecco's phosphate-buffered saline and then frozen in liquid nitrogen and stored at –80°C. When required, these were thawed on ice and scraped into hypotonic lysis buffer (50 mM Tris–HCl, 3 mM ethylenediaminetetraacetic acid, 3 mM ethylene glycol bis(2-aminoethyl ether)-$N,N,N'N'$-tetraacetic acid, 0.5 mM NaF, 10 mM β-glycerophosphate, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 0.1% β-mercaptoethanol; pH 7.4) at 4°C. Cytoplasmic and membrane portions were separated by centrifugation at $10\,000 \times g$ for 10 min at 4°C. After estimating protein concentration using the BioRad protein assay, samples were solved in Laemmli buffer, heated to 100°C and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore Corp. Bedford, MA, USA). Membranes were blocked with TBST + 5% milk or BSA, and specific proteins were detected by incubation with the appropriate primary and horseradish-peroxidase-conjugated secondary antibodies. Bound antibodies were detected by enhanced chemiluminescence. β-catenin levels were quantified from band density on Western blots using NIH Picture Viewer software.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from cultured cells using an RNeasy kit (Qiagen, Crawley, UK) and mRNA expression was analysed by RT-PCR as described previously.[28] Primers and probes for C/EBPα were purchased from Applied Biosystems. Primers and probes for murine PPARγ2, aP2, PPARδ, C/EBPβ, c-myc, cyclin D1, GATA2, GATA3, Wnt10b and β-catenin were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The sequences of these oligonucleotides are available upon request.

Apoptosis and cell viability assays

Analysis of TNF-α-induced apoptosis was based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL). The assay was performed using the *in situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, Burgess Hill, UK) according to the manufacturer's instructions. The extent of apoptosis was then determined by flow cytometric analysis of a single cell suspension using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser and emission wavelength of 488 nm. 10 000 events were collected and the percentage of TUNEL-positive cells was analysed using FlowJo 8.2 software (FlowJo LLC, Ashland, OR, USA).

Statistical analysis

Data from densitometrical analysis, luciferase assays and quantitative real-time PCR are presented as mean±S.E.M. of at least three independent experiments. Statistical significance was tested using a paired two-tailed Student's t-test assuming equal variances. Unless

otherwise stated, statistical significance is reported at each time point for −TNF-*α versus* +TNF-*α* samples, as follows: *P<0.05; **P<0.01; ***P<0.001.

Acknowledgements

Abbreviations

TNF-*α*	tumour necrosis factor-alpha
TNFR1	TNF-*α* receptor-1
PPAR	peroxisome proliferators-activated receptor
C/EBP	CCAAT/enhancer-binding protein
TCF4	T-cell factor 4 (also known as TCF7L2)
NF-*κ*B	nuclear factor kappa B

References

1. Farmer SR. Transcriptional control of adipocyte formation. Cell Metab. 2006; 4:263–273. [PubMed: 17011499]

2. MacDougald OA, Mandrup S. Adipogenesis: forces that tip the scales. Trends Endocrinol Metab. 2002; 13:5–11. [PubMed: 11750856]

3. Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, et al. Inhibition of adipogenesis by Wnt signaling. Science. 2000; 289:950–953. [PubMed: 10937998]

4. Xu H, Sethi JK, Hotamisligil GS. Transmembrane tumor necrosis factor (TNF)-alpha inhibits adipocyte differentiation by selectively activating TNF receptor 1. J Biol Chem. 1999; 274:26287–26295. [PubMed: 10473584]

5. Huelsken J, Behrens J. The Wnt signalling pathway. J Cell Sci. 2002; 115:3977–3978. [PubMed: 12356903]

6. Moldes M, Zuo Y, Morrison RF, Silva D, Park BH, Liu J, et al. Peroxisome-proliferator-activated receptor gamma suppresses Wnt/beta-catenin signalling during adipogenesis. Biochem J. 2003; 376:607–613. [PubMed: 12954078]

7. Yang X, Jansson PA, Nagaev I, Jack MM, Carvalho E, Sunnerhagen KS, et al. Evidence of impaired adipogenesis in insulin resistance. Biochem Biophys Res Commun. 2004; 317:1045–1051. [PubMed: 15094374]

8. Sethi JK, Vidal-Puig AJ. Thematic Review Series on Adipocyte Biology: Adipose tissue function and plasticity orchestrate nutritional adaptation. J Lipid Res. 2007 in press.

9. Xu H, Hotamisligil GS. Signaling pathways utilized by tumor necrosis factor receptor 1 in adipocytes to suppress differentiation. FEBS Lett. 2001; 506:97–102. [PubMed: 11591379]

10. Chae GN, Kwak SJ. NF-kappaB is involved in the TNF-alpha induced inhibition of the differentiation of 3T3-L1 cells by reducing PPARgamma expression. Exp Mol Med. 2003; 35:431–437. [PubMed: 14646597]

11. Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, Yamauchi T, et al. Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. Nat Cell Biol. 2003; 5:224–230. [PubMed: 12598905]

12. Berg AH, Lin Y, Lisanti MP, Scherer PE. Adipocyte differentiation induces dynamic changes in NF-kappaB expression and activity. Am J Physiol Endocrinol Metab. 2004; 287:E1178–1188. [PubMed: 15251865]

13. Castro-Munozledo F, Beltran-Langarica A, Kuri-Harcuch W. Commitment of 3T3-F442A cells to adipocyte differentiation takes place during the first 24-36 h after adipogenic stimulation: TNF-alpha inhibits commitment. Exp Cell Res. 2003; 284:163–172. [PubMed: 12651150]

14. Morrison RF, Farmer SR. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J Biol Chem. 1999; 274:17088–17097. [PubMed: 10358062]

15. Burton GR, Nagarajan R, Peterson CA, McGehee RE Jr. Microarray analysis of differentiation-specific gene expression during 3T3-L1 adipogenesis. Gene. 2004; 329:167–185. [PubMed: 15033539]

16. Tong Q, Dalgin G, Xu H, Ting CN, Leiden JM, Hotamisligil GS. Function of GATA transcription factors in preadipocyte–adipocyte transition. Science. 2000; 290:134–138. [PubMed: 11021798]

17. Ninomiya-Tsuji J, Torti FM, Ringold GM. Tumor necrosis factor-induced c-myc expression in the absence of mitogenesis is associated with inhibition of adipocyte differentiation. Proc Natl Acad Sci USA. 1993; 90:9611–9615. [PubMed: 8415749]

18. Freytag S, Geddes TJ. Reciprocal regulation of adipogenesis by Myc and C/EBPalpha. Science. 1992; 256:379–382. [PubMed: 1566086]

19. Fu M, Rao M, Bouras T, Wang C, Wu K, Zhang X, et al. Cyclin D1 inhibits peroxisome proliferator-activated receptor gamma-mediated adipogenesis through histone deacetylase recruitment. J Biol Chem. 2005; 280:16934–16941. [PubMed: 15713663]

20. Shi Y, Hon M, Evans RM. The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. PNAS. 2002; 99:2613–2618. [PubMed: 11867749]

21. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a target of the APC pathway. Science. 1998; 281:1509–1512. [PubMed: 9727977]

22. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, et al. The cyclin D1 gene is a target of the beta -catenin/LEF-1 pathway. PNAS. 1999; 96:5522–5527. [PubMed: 10318916]

23. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell. 1999; 99:335–345. [PubMed: 10555149]

24. Hagen T, Sethi JK, Foxwell N, Vidal-Puig A. Signalling activity of beta-catenin targeted to different subcellular compartments. Biochem J. 2004; 379:471–477. [PubMed: 14733614]

25. Kolligs FT, Hu G, Dang CV, Fearon ER. Neoplastic transformation of RK3E by mutant beta-catenin requires deregulation of Tcf/Lef transcription but not activation of c-myc expression. Mol Cell Biol. 1999; 19:5696–5706. [PubMed: 10409758]

26. Bennett CN, Ross SE, Longo KA, Bajnok L, Hemati N, Johnson KW, et al. Regulation of Wnt signaling during adipogenesis. J Biol Chem. 2002; 277:30998–31004. [PubMed: 12055200]

27. Gustafson B, Smith U. Cytokines promote WNT signaling and inflammation and impair the normal differentiation and lipid accumulation in 3T3-L1 preadipocytes. J Biol Chem. 2006; 281:9507–9516. [PubMed: 16464856]

28. Christodoulides C, Laudes M, Cawthorn WP, Schinner S, Soos M, O'Rahilly S, et al. The Wnt antagonist Dickkopf-1 and its receptors are coordinately regulated during early human adipogenesis. J Cell Sci. 2006; 119:2613–2620. [PubMed: 16763196]

29. Wu Z, Rosen ED, Brun R, Hauser S, Adelmant G, Troy AE, et al. Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. Mol Cell. 1999; 3:151–158. [PubMed: 10078198]

30. Chen S, Guttridge DC, You Z, Zhang Z, Fribley A, Mayo MW, et al. Wnt-1 signaling inhibits apoptosis by activating beta-catenin/T cell factor-mediated transcription. J Cell Biol. 2001; 152:87–96. [PubMed: 11149923]

31. Orford K, Orford CC, Byers SW. Exogenous expression of beta-catenin regulates contact inhibition, anchorage-independent growth, anoikis, and radiation-induced cell cycle arrest. J Cell Biol. 1999; 146:855–868. [PubMed: 10459019]

32. Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, et al. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet. 1998; 19:379–383. [PubMed: 9697701]

33. Cauchi S, Meyre D, Dina C, Choquet H, Samson C, Gallina S, et al. Transcription factor TCF7L2 genetic study in the French population: expression in human beta-cells and adipose tissue and strong association with type 2 diabetes. Diabetes. 2006; 55:2903–2908. [PubMed: 17003360]

34. Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Sainz J, et al. Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat Genet. 2006; 38:320–323. [PubMed: 16415884]

35. Saxena R, Gianniny L, Burtt NP, Lyssenko V, Giuducci C, Sjogren M, et al. Common single nucleotide polymorphisms in TCF7L2 are reproducibly associated with type 2 diabetes and reduce the insulin response to glucose in nondiabetic individuals. Diabetes. 2006; 55:2890–2895. [PubMed: 17003358]

36. Sharma C, Pradeep A, Wong L, Rana A, Rana B. Peroxisome proliferator-activated receptor-gamma activation can regulate beta-catenin levels via a proteasome-mediated and Adenomatous Polyposis Coli-independent pathway. J Biol Chem. 2004; 279:35583–35594. [PubMed: 15190077]

37. Liu J, Farmer SR. Regulating the balance between peroxisome proliferator-activated receptor gamma and beta-catenin signaling during adipogenesis. A glycogen synthase kinase 3beta phosphorylation-defective mutant of beta-catenin inhibits expression of a subset of adipogenic genes. J Biol Chem. 2004; 279:45020–45027. [PubMed: 15308623]

38. Lamberti C, Lin KM, Yamamoto Y, Verma U, Verma IM, Byers S, et al. Regulation of beta-catenin function by the IkappaB kinases. J Biol Chem. 2001; 276:42276–42286. [PubMed: 11527961]

39. Carayol N, Wang CY. IKKalpha stabilizes cytosolic beta-catenin by inhibiting both canonical and non-canonical degradation pathways. Cell Signal. 2006; 18:1941–1946. [PubMed: 16616828]

40. Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, et al. Erk Associates with and Primes GSK-3beta for Its Inactivation Resulting in Upregulation of beta-Catenin. Mol Cell. 2005; 19:159–170. [PubMed: 16039586]

41. Tran SE, Holmstrom TH, Ahonen M, Kahari VM, Eriksson JE. MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. J Biol Chem. 2001; 276:16484–16490. [PubMed: 11278665]

42. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. Cell. 1993; 74:845–853. [PubMed: 8397073]

43. Boone E, Vanden Berghe T, Van Loo G, De Wilde G, De Wael N, Vercammen D, et al. Structure/Function analysis of p55 tumor necrosis factor receptor and fas-associated death domain. Effect on necrosis in L929sA cells. J Biol Chem. 2000; 275:37596–37603. [PubMed: 10988295]

44. Jiang Y, Woronicz JD, Liu W, Goeddel DV. Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. Science. 1999; 283:543–546. [PubMed: 9915703]

45. Nawaratne R, Gray A, Jorgensen CH, Downes CP, Siddle K, Sethi JK. Regulation of insulin receptor substrate 1 pleckstrin homology domain by protein kinase C: role of serine 24 phosphorylation. Mol Endocrinol. 2006; 20:1838–1852. [PubMed: 16574739]
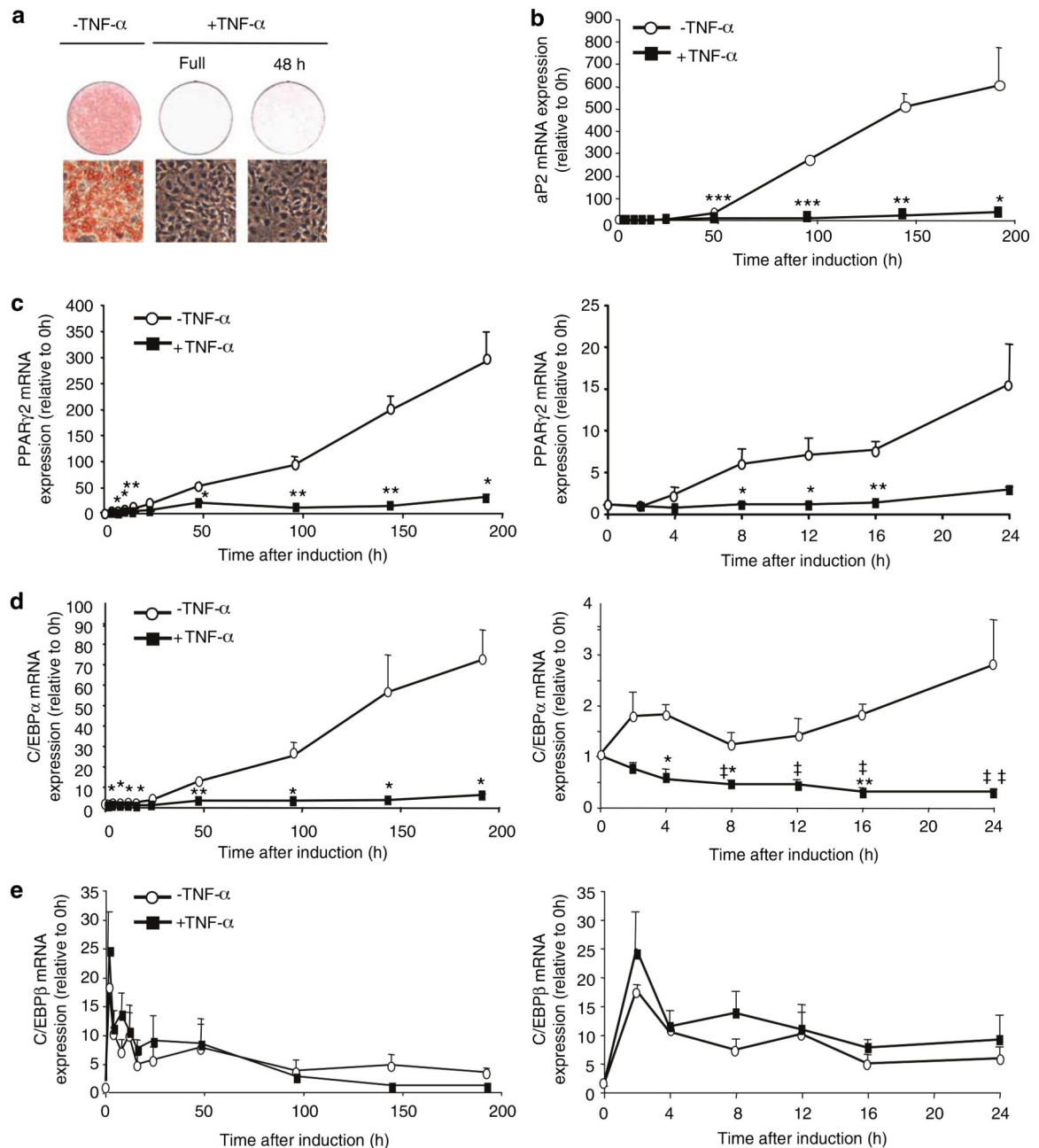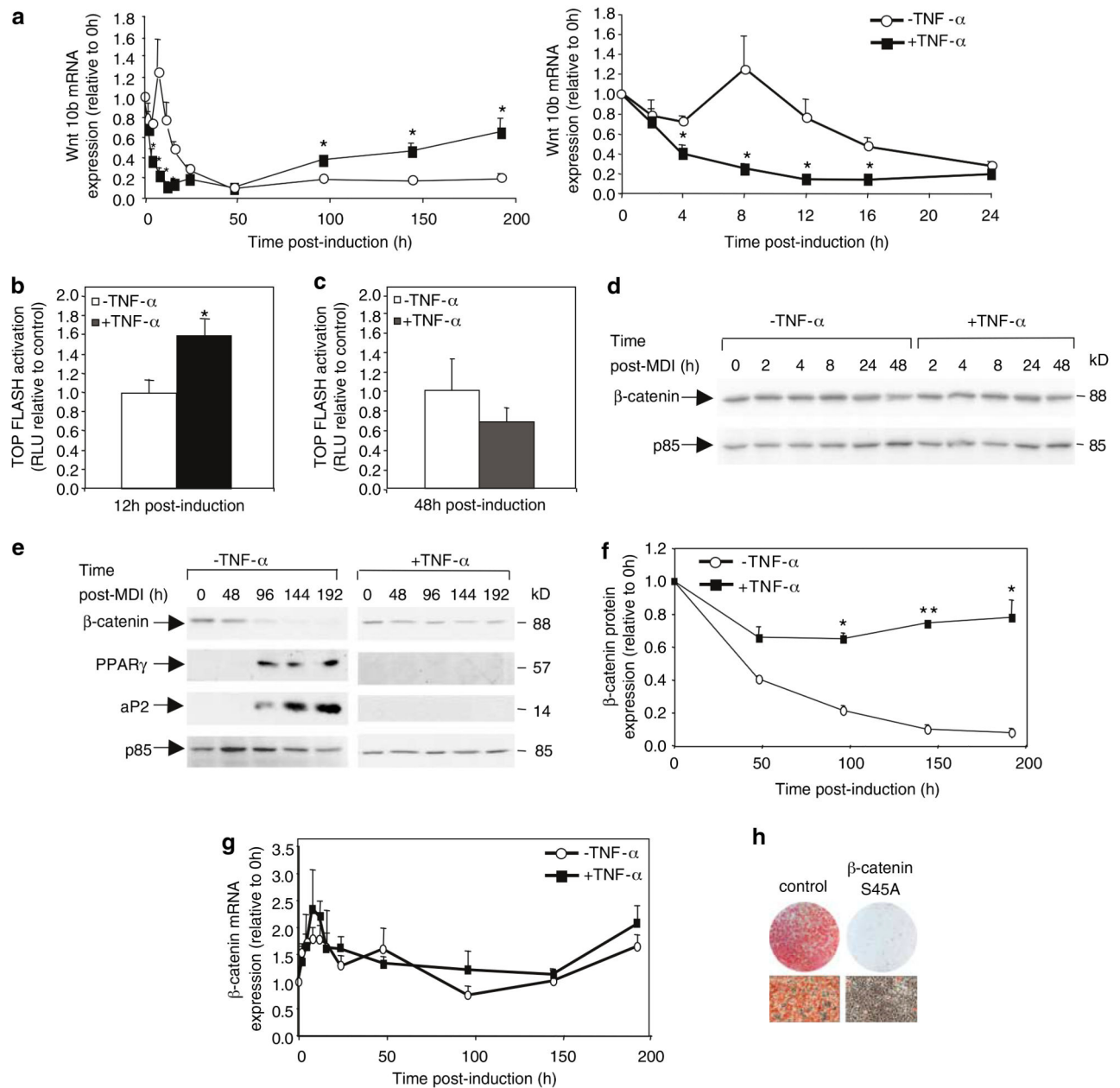
Figure 1.

Short-term exposure to TNF-α is sufficient to inhibit adipogenesis in 3T3-L1 cells.
Confluent 3T3-L1 preadipocytes were induced to differentiate as described in *Materials and Methods*. Cells were treated with or without TNF-α (2 ng/ml) either throughout the differentiation procedure (Full) or for the first 48 h after induction (48 h). (**a**) At 8 days post-induction, the extent of adipogenesis was assessed by staining for lipid accumulation with Oil Red O. The data are representative of five independent experiments. (**b–e**) 3T3-L1 cells were induced to differentiate with or without TNF-α (2 ng/ml for the first 48 h) and total RNA was extracted at 0, 2, 4, 8, 12, 16, 24, 48, 96, 144 and 192 h post-induction. Transcript

levels of aP2 (**b**), PPARγ2 (**c**), C/EBPα (**d**) and C/EBPβ (**e**) were determined by using real-time PCR and normalised to 18S rRNA. Gene expression is reported relative to levels at 0 h as mean±S.E.M. of three independent experiments. Statistical significance is indicated as follows: Compared to control treatment at the same time point (*P<0.05; **P<0.01; ***P<0.001); TNF treatment compared to time 0 ($\ddagger$ = P<0.05; $\ddagger\ddagger$ = P<0.01)
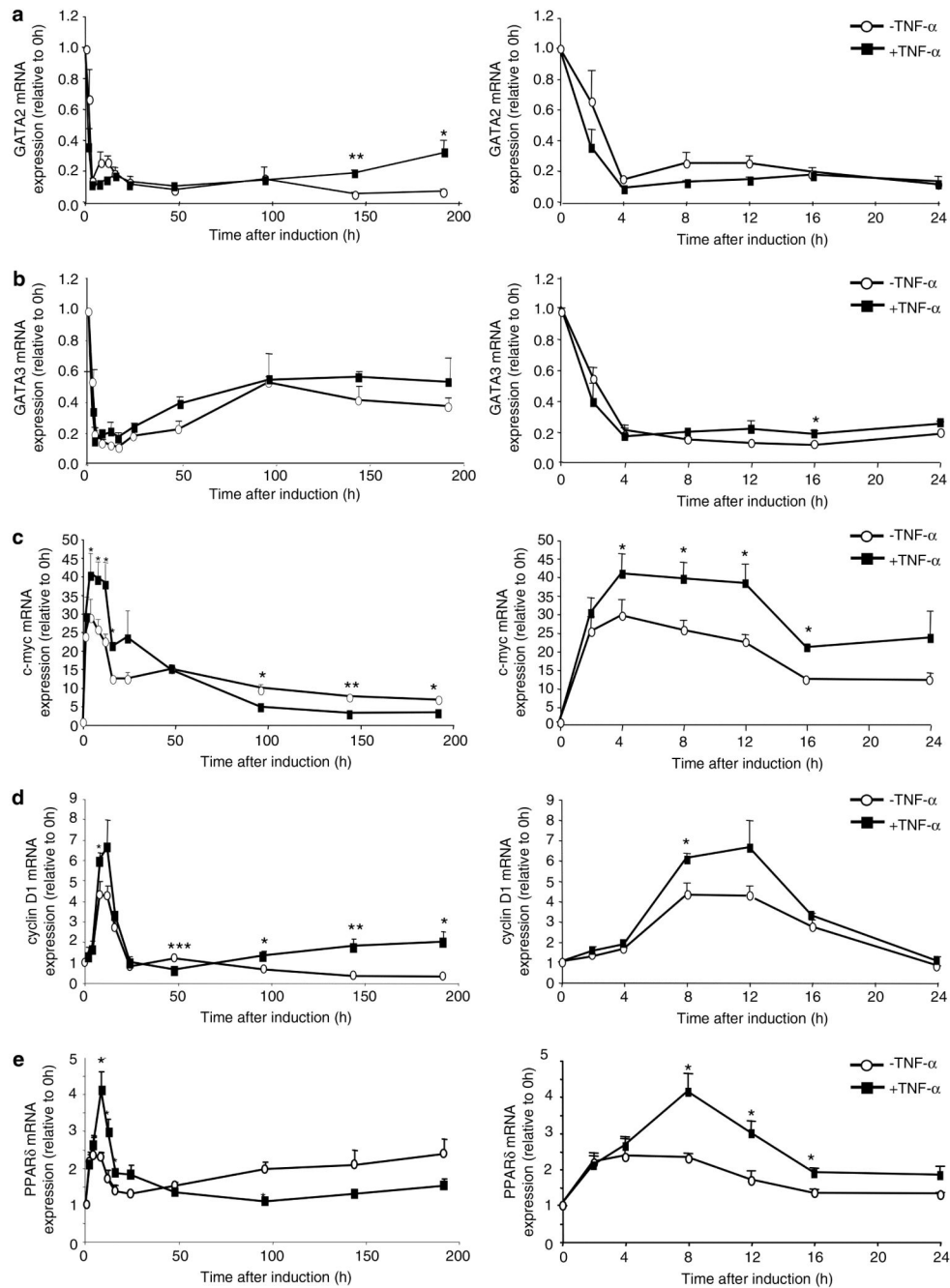
Figure 2.
Effect of TNF-α treatment on the expression of antiadipogenic mediators during the early stages of antiadipogenesis. RNA samples were collected during 3T3-L1 adipogenesis and antiadipogenesis as described in Figure 1. Expression of GATA2 (**a**), GATA3 (**b**), c-myc (**c**), cyclin D1 (**d**) and PPARδ (**e**) was determined by using real-time PCR and normalised to 18S rRNA. Gene expression and statistically significant differences are reported as described in Figure 1
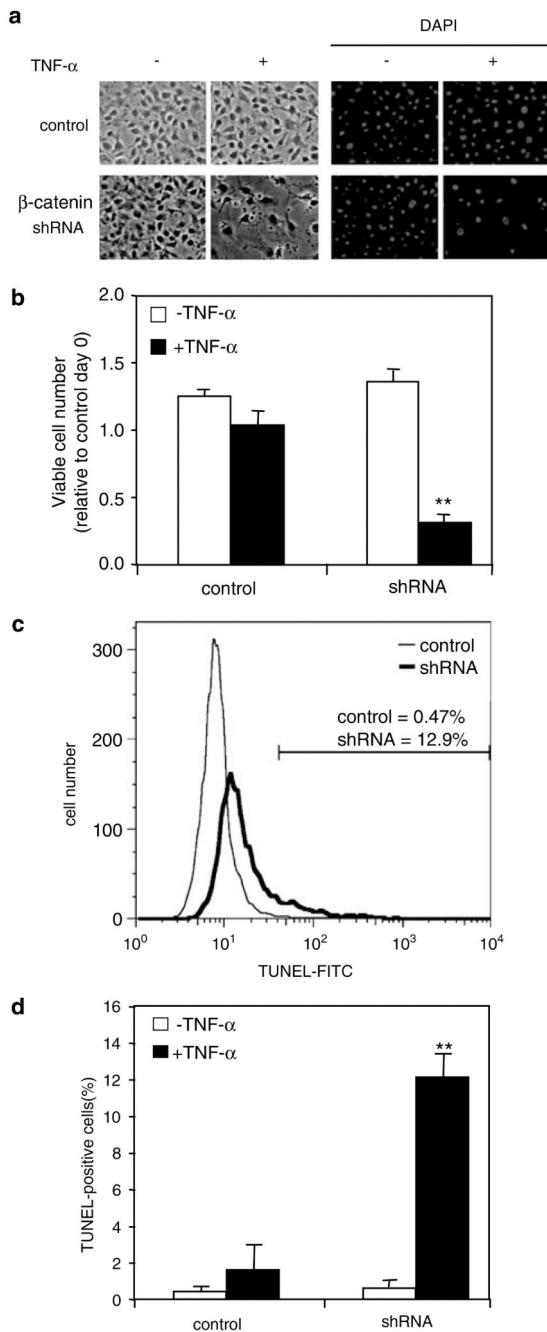
Figure 3.

TNF-α activates β-catenin/TCF signalling during early antiadipogenesis. Expression of Wnt10b (**a**) was determined as described in Figures 1 and 2. (**b** and **c**) 3T3-L1 preadipocytes were also transfected with the TCF reporter Topflash as described in *Materials and Methods*. Three days post-transfection, cells were induced to differentiate in the absence or presence of TNF-α (2 ng/ml). Luciferase activity was assayed 12 h (**b**) or 48 h (**c**) post-induction. The results are reported relative to luciferase activity in the absence of TNF-α as mean±S.E.M. of five (**b**) or four (**c**) independent experiments, with each experiment carried out in duplicate. (**d**, **e** and **f**) Confluent 3T3-L1 preadipocytes were induced to differentiate with or without TNF-α (for the first 48 h after induction) and cytosolic protein was extracted

at the indicated times post-induction. Expression of β-catenin, aP2, and PPARγ was then analysed by Western blotting. Time 0 h proteins were extracted before differentiation was induced. The p85 subunit of PI3K was used as a loading control. Quantification of β-catenin levels from (**e**) by densitometrical analysis and normalisation to p85 levels is shown in (**f**) and is expressed as mean±S.E.M. of three independent experiments. (**g**) β-catenin mRNA expression during adipogenesis and TNF-α-induced antiadipogenesis. Total RNA was extracted as described in Figure 1. (**h**) 3T3-L1 preadipocytes expressing β-catenin S45A or an empty vector were induced to differentiate with MDI. The extent of adipogenesis at 8 days post-induction was assessed by staining for lipid accumulation with oil red O
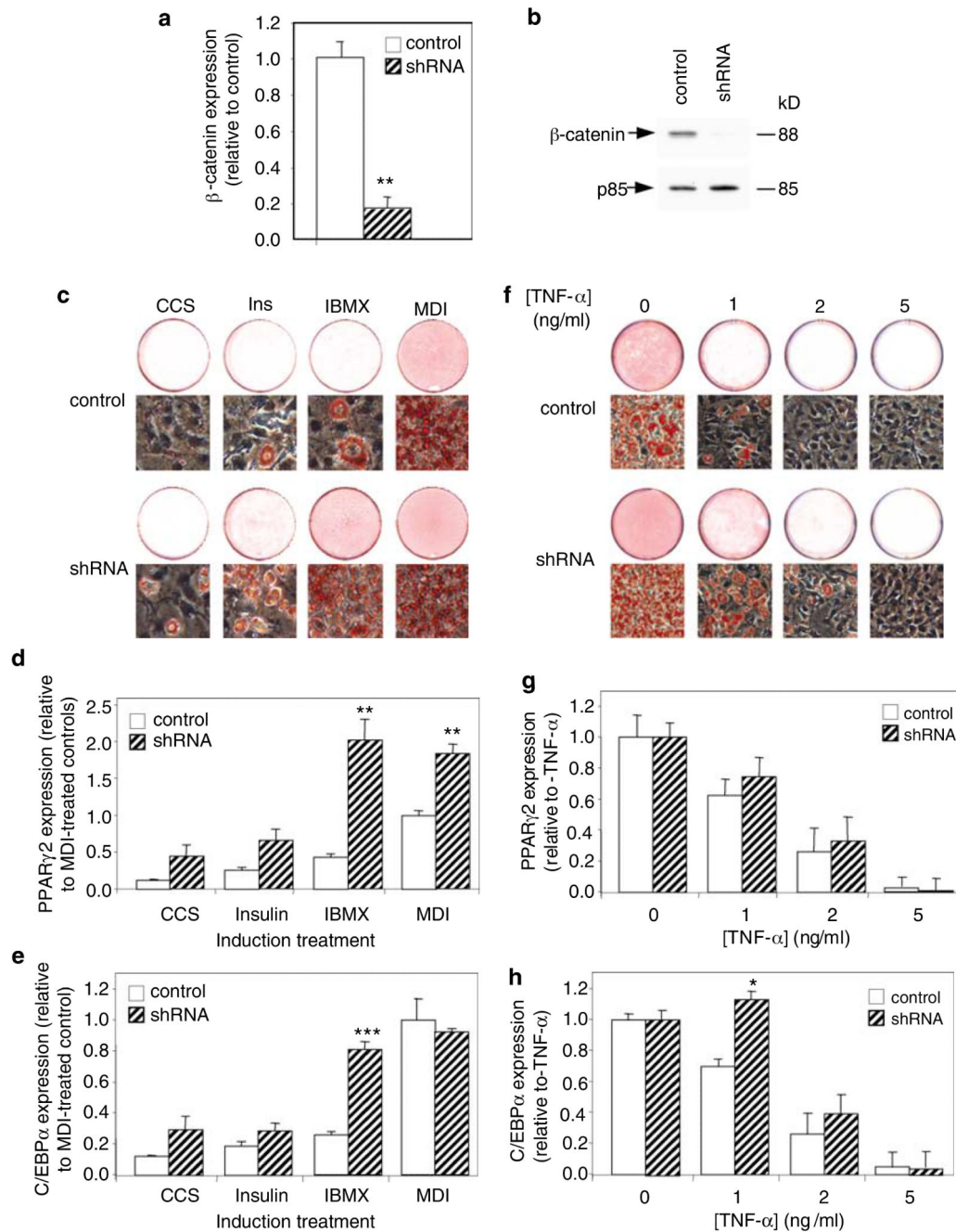
Figure 4.

Knockdown of β-catenin enhances adipogenesis in 3T3-L1 preadipocytes and attenuates TNF-α-induced antiadipogenesis. 3T3-L1 preadipocytes were transfected with vectors encoding shRNAs against firefly luciferase (control) or murine β-catenin (shRNA). (**a**) Total RNA was extracted from confluent control and β-catenin shRNA 3T3-L1 preadipocytes and expression of β-catenin mRNA was determined by using real-time PCR and normalised to 18S rRNA. (**b**) Cytosolic protein was extracted from confluent 3T3-L1 preadipocytes expressing control or β-catenin shRNA and expression of β-catenin protein was analysed by

Western blotting. p85 was used as a loading control. The result shown is representative of four independent experiments. (**c–e**) Control and β-catenin shRNA cells were induced to differentiate with serum only (cosmic calf serum – CCS), insulin (Ins), IBMX or MDI. The extent of adipogenesis at 8 days post-induction was assessed by oil red O staining (**c**) or extraction of total RNA followed by real-time PCR analysis of PPARγ2 (**d**) and C/EBPα (**e**) mRNA expression. (**f–h**) Control and β-catenin shRNA cells were induced to differentiate with MDI in the presence of the indicated concentrations of TNF-α, which were maintained in the adipogenic media for the first 48 h post-induction only. The extent of adipogenesis at 8 days post-induction was assessed by oil red O staining (**f**) or extraction of total RNA followed by real-time PCR analysis of PPARγ2 (**g**) and C/EBPα (**h**) mRNA expression. Results in (**a**), (**d**) and (**e**), (**g**) and (**h**) are reported relative to levels in control cells as mean ±S.E.M. of three independent experiments. The results in (**c**) and (**f**) are representative of three independent experiments. For each induction treatment, statistically significant differences between control and β-catenin knockdown cells are reported as follows: *P<0.05; **P<0.01; ***P<0.001

Figure 5.
Knockdown of β-catenin promotes apoptosis during TNF-α-induced antiadipogenesis.
Control and β-catenin shRNA 3T3-L1 preadipocytes were induced to differentiate with or
without TNF-α (2 ng/ml). At 0 h and 24 h post-induction, cells were either fixed and stained
with DAPI and analysed by fluorescence microscopy, or analysed for DNA fragmentation
by TUNEL. (**a**) Micrographs of cells at 24 h post-induction. (**b**) Quantification of viable,
adherent cell number as indicated by DAPI staining of adherent cells. (**c**) Flow cytometric
analysis of TUNEL staining in control and β-catenin shRNA 3T3-L1 cells at 24 h post-

induction in the presence of TNF-α. Shown is a representative histogram. (**d**) The percentage of TUNEL-positive cells (indicated in (**c**)) as assessed by FACS analysis. The results in (**a**) and (**c**) are representative of three independent experiments. The results in (**b**) and (**d**) are expressed as mean±S.E.M. of three independent experiments. Statistically significant differences between −TNF-α and +TNF-α samples are reported as described for Figure 1
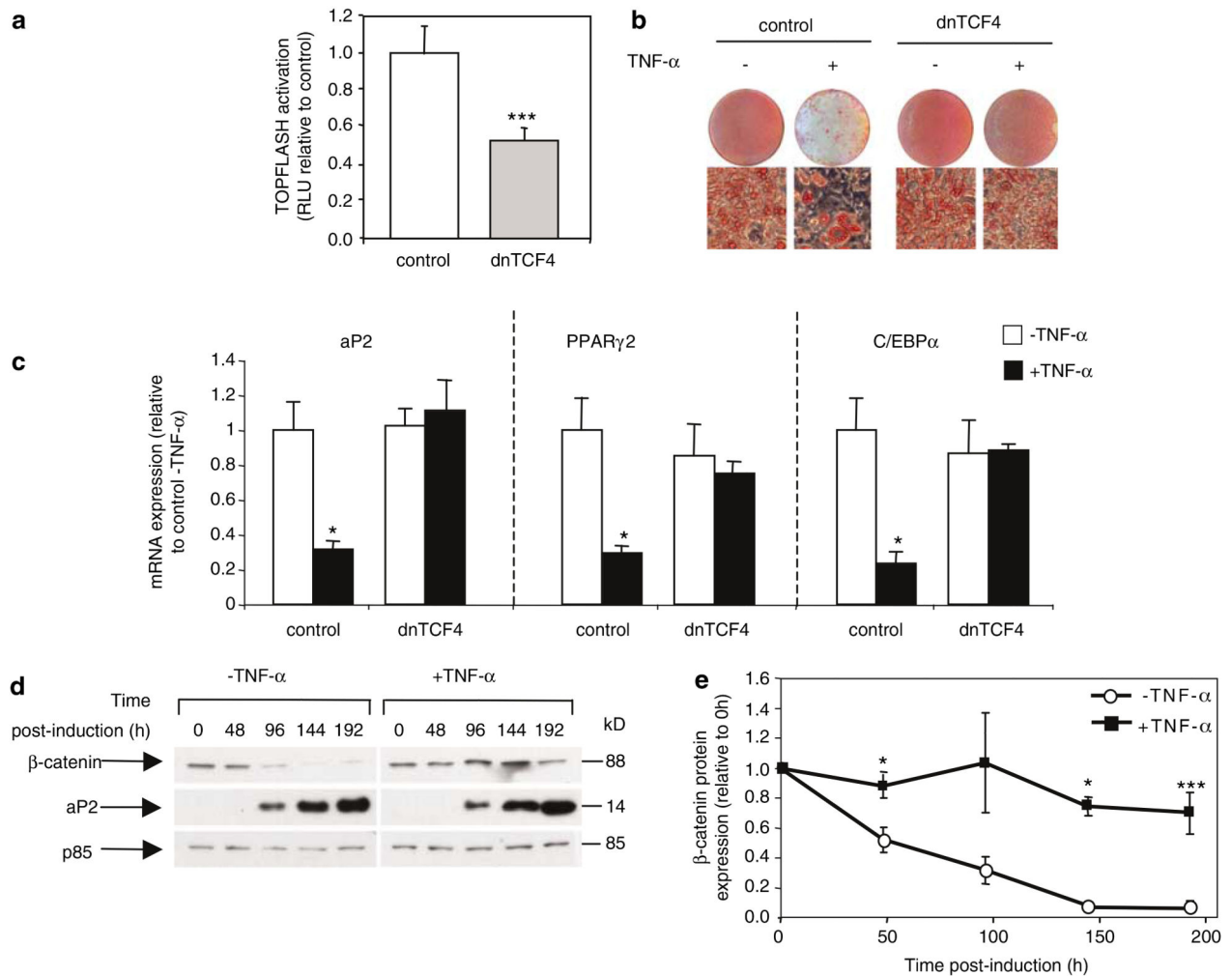
Figure 6.

DnTCF4 reverses TNF-α-induced antiadipogenesis but not TNF-α-induced stabilisation of β-catenin. (**a**) 3T3-L1 preadipocytes expressing dnTCF4 or a control vector were transfected with the TCF reporter Topflash as described in *Materials and Methods*. Three days post-transfection, cells were induced to differentiate and luciferase activity was assayed at 12 h post-induction. The results are reported relative to those in control cells as mean±S.E.M. of two independent experiments, with each experiment done in triplicate. (**b–e**) Control and dnTCF4 cells were induced to differentiate in the absence or presence of TNF-α (1 ng/ml). (**b**) Oil red O-stained cells at 8-days post-induction of differentiation. (**c**) Total RNA was extracted at 8 days post-induction and the expression of aP2, PPARγ2 and C/EBPα mRNA was analysed by real-time PCR and normalised to 18S rRNA. (**d**) Cytosolic protein was extracted at the indicated times post-induction. Expression of β-catenin and aP2 was then analysed by Western blotting. Time 0 h proteins were extracted before differentiation was induced. p85 was used as a loading control. (**e**) Quantification of β-catenin levels from (**d**) by densitometrical analysis and normalisation to p85 levels to correct for variations in loading protein concentration. The results in (**b**) and (**d**) are representative of three independent experiments. The results in (**c**) and (**e**) are reported as mean±S.E.M. of three
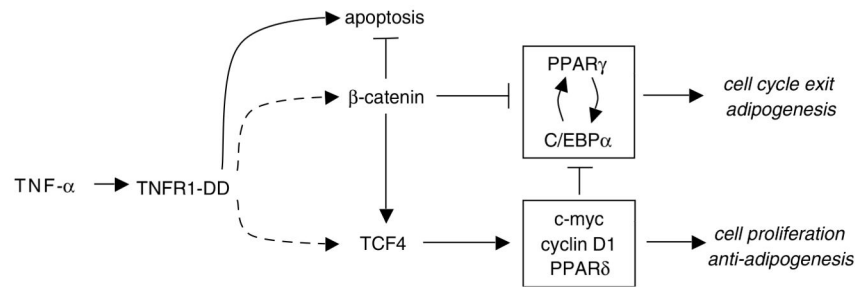
independent experiments. Statistically significant differences between $-$TNF-α and $+$TNF-α samples are reported as described in Figure 1
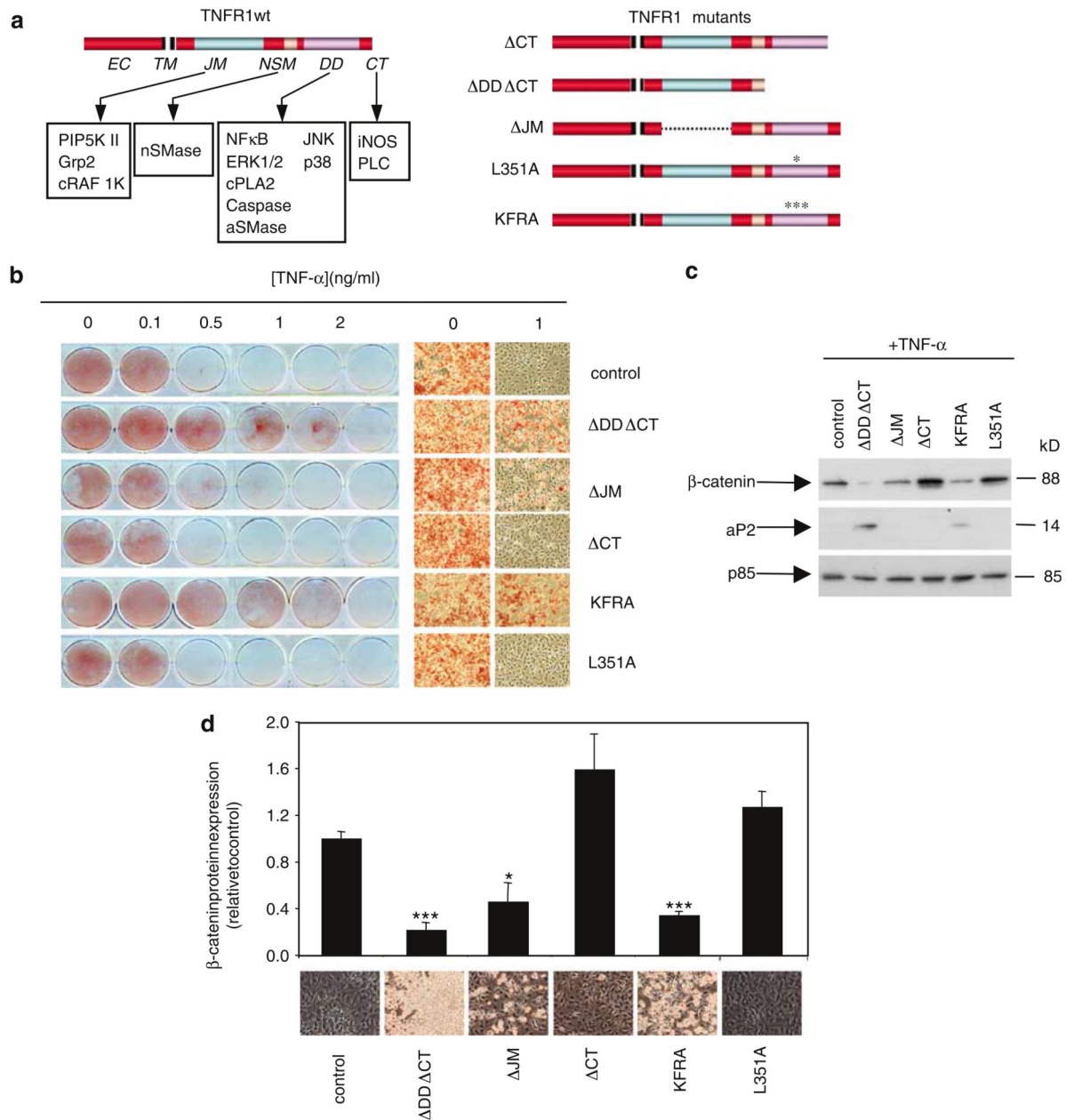
Figure 7.

A functional death domain of TNFR1 is required for antiadipogenesis and *β*-catenin stabilisation by TNF-*α*. (**a**) Schematic representation of the domains of TNFR1, the signalling pathways activated via these domains and the alterations of these domains in each TNFR1 mutant (as described in *Materials and Methods*). (**b**) 3T3-L1 preadipocytes overexpressing TNFR1 mutants or an empty vector control were induced to differentiate in the absence or presence of TNF-*α*, as indicated. The extent of differentiation at 10 days post-induction was assessed by oil red O staining. Plates of cells (35 mm) and micrographs are shown. (**c**) Western blots showing the expression of *β*-catenin and aP2 at 8 days post-induction of adipogenesis in the presence of 2 ng/ml TNF-*α*. p85 was used as a loading

control. The results in (**b**) and (**c**) are representative of three independent experiments. (**d**) Quantification of β-catenin levels from (**c**) by densitometrical analysis and normalisation to p85 levels to correct for variations in loading protein concentration. Representative micrographs of unstained cells are shown below the graph for each cell type. The results are reported as mean±S.E.M. of three independent experiments. Statistically significant differences between control and TNFR1 mutant-overexpressing cells are reported as follows: *$P<0.05$; **$P<0.01$; ***$P<0.001$