The role of Tip60 in adipogenesis

De rol van Tip60 in vetcel differentiatie

(met een samenvatting in het Nederlands)

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Chapter 1

GENERAL INTRODUCTION

1.1 Energy metabolism

The existence of an organism depends on the continuous provision of energy to drive metabolic processes. This is what we call energy metabolism, which is one of the most obvious differences between life and non-life. There are three main energy sources: carbohydrates, fats and protein. The main metabolic fuels are glucose and fatty acids. Carbohydrates are metabolized to glucose, which is the primary source of energy used in the body and it is stored as glycogen. In normal circumstances, glucose is the only fuel which can be used by the brain and it is estimated that 55-60 percent of energy is coming from digestion of carbohydrates. The esters of glycerol and long-chain fatty acids, called triglycerides or triacylglycerol (TG), are the ideal storage of fuel. Amino acids can be used as a fuel during fasting, illness, or injury.
Energy metabolism could also viewed as a stimulus-response process of the energy resources (food) with the organism: how does the organism respond to the intake of different nutrients? Here the metabolism of two main energy sources will be discussed: glucose and fatty acids (Fig. 1).

Glucose homeostasis

Carbohydrates digestion starts with amylase in the mouth, but the key digestion occurred in the intestine by the enzymes secreted by pancreas. After the breakdown and absorption in the intestine, most of the products are delivered to the liver, transformed to glucose and released into bloodstream (Fig. 1). Plasma glucose concentrations are maintained in a relatively narrow range throughout the day and this is realized by the counteraction of two major hormones: the anabolic hormone insulin and the catabolic hormone glucagon. Together they control the balance between intake (glucose absorption from the gut), tissue utilization (glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, glycogen synthesis) and endogenous production (glycogenolysis and gluconeogenesis). Insulin and glucagon are produced by the pancreatic β- and α-cells, respectively. In response to an increase in plasma glucose following a meal, insulin decreases the plasma glucose concentration by promoting (i) the uptake of glucose into tissues, (ii) intracellular glucose metabolism, and (iii) glycogen synthesis. Glucagon stimulates both the release of glucose from glycogen stores and its *de novo* synthesis, thus causing an increase in plasma glucose concentration. The balance between the action of

insulin and glucagon is the key factor in the control of glucose metabolism.

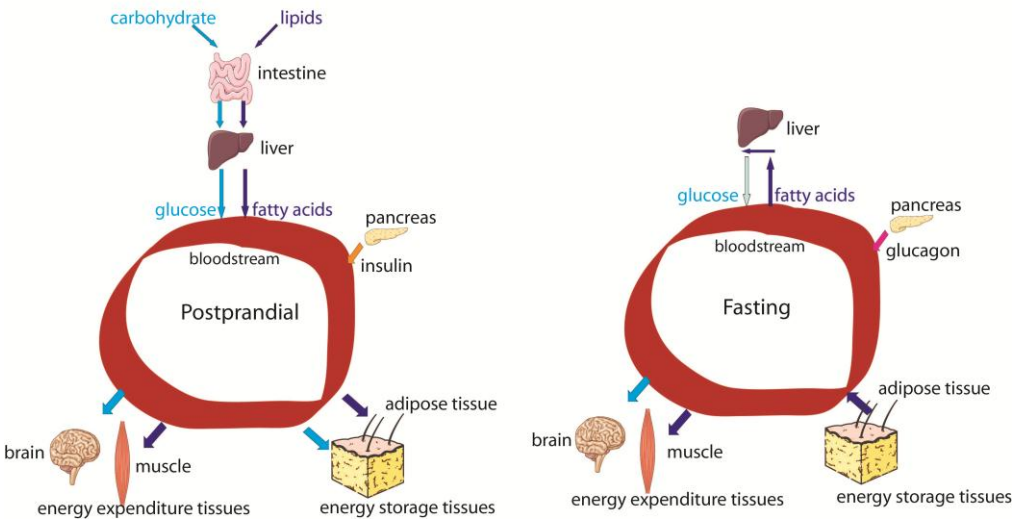

Figure 1. Schematic representation of carbohydrate and lipid metabolism in the postprandial state (after a meal) and in the fasting state.

Lipid metabolism

Dietary lipids first have to be emulsified and then be degraded by pancreatic lipase (Fig. 1). These enzymes generate free fatty acids and a mixture of mono- and diacylglycerols from dietary triacylglycerols. Following absorption of the products of pancreatic lipase by the intestinal mucosal cells, the resynthesis of triacylglycerols occurs. The triacylglycerols are then solubilized in lipoprotein complexes (complexes of lipid and protein) called chylomicrons. A chylomicron contains lipid droplets surrounded by more polar lipids and finally a layer of proteins. Triacylglycerols synthesized in the liver are packaged into VLDLs and released into the blood directly. Chylomicrons from the intestine are then released into the blood via the lymph system for delivery to the various tissues for storage or production of energy through oxidation.

Adipocytes at the cross-roads of metabolic regulation

Besides playing an important role in providing insulation and protection against mechanical stress, white adipose tissue (WAT) has

long been recognized as a storage depot for excess energy [1]. FFA are released from VLDL particles, re-esterified and stored as triglycerides in large lipid droplets in white adipocytes, and it this way WAT not only stores energy but also buffers other organs from overexposure to FFA. In time of caloric need lipases will hydrolyze tissues to generate energy [1]. Recently, WAT has been recognized as an endocrine organ, releasing a wide range of adipokines, which for example regulate immune responses, blood pressure control, angiogenesis, haemostasis, bone mass and thyroid and reproductive function [2,3]. Expansion of white adipose tissue, as seen during the development of increased bodyweight and obesity, involves an increase in adipocyte size and the formation of new adipocytes from precursor cells (adipogenesis).

Brown adipose tissue (BAT) is specialized for non-shivering thermogenesis, the process whereby the energy derived from fatty acid oxidation is used for the generation of heat due to mitochondrial uncoupling [4]. In brown adipocytes triglycerides are stored in multiple small lipid droplets, and there is a high content of mitochondria [4]. For a long time it was assumed that in humans BAT was mainly present in infants and relatively scarce in adults [5]. In contrast, small mammals (e.g. rodents), even adults, have brown adipose tissue to defend them against the cold [6]. Recently human adults were also shown to have distinct BAT depots [7,8,9].

Together with for example bone cells (osteocytes) and muscle cells (myocytes), white and brown adipocytes both are derived from mesenchymal stem cells (MSC). Precursor cells of WAT reside in the mural cell compartments of the adipose vasculature (reviewed in [10]). Lineage-tracing experiments in mice have shown that brown adipocytes are derived from Myf5-positive progenitor cells, which also give rise to myocytes, while white adipocytes are Myf5-negative [11]. Brown adipocytes have been detected in WAT after chronic cold exposure or β-adrenergic stimulation [12] and these cells did not originate from the Myf5-positive progenitor cells [11]. Such brown adipocytes, which clearly have a distinct developmental origin from brown adipocytes in BAT, are sometimes referred to as “brite” or “beige” adipocytes [13]. It should be noted that it is currently debated whether “brite” or “beige” adipocytes may also arise from transdifferentiation of white adipocytes, as can be observed under certain experimental conditions *in vitro* [14].

Multiple lines of evidence indicate that proper function of white adipocytes and adipose tissue is required for metabolic health. Most

importantly, increased body weight and obesity are associated with serious complications, such as insulin resistance, type 2 diabetes, hepatic steatosis, dyslipidemia and cardiovascular disease. While the pathways leading from obesity to its complications are multifold [15,16,17], two phenomena may help to explain the central role of adipocytes and adipose tissue in energy homeostasis. First, the storage capacity of adipose tissue may be exceeded at some point under conditions of excessive food intake. This will result in so-called ectopic storage of lipids in non-adipose tissue, like liver and muscle, which plays in important role in the development of insulin resistance in these tissues [15]. In addition, expansion of adipose tissue is associated with qualitative and quantitative changes in a number of secreted adipokines, which affect metabolic functions of other organs [3,18].

Adipogenesis

Since adipose tissue is increasingly being recognized as a key regulator of whole-body energy homeostasis and consequently as a prime therapeutic target for metabolic syndrome, adipocyte differentiation and biology are under intensive study. Many of the molecular mechanisms underlying adipocyte differentiation have been discovered using various preadipocyte cell culture systems, the 3T3-L1 preadipocyte cell line being the best-known [19]. 3T3-L1 is a clonal cell line derived from mouse 3T3 cells, selected on basis of its ability to differentiate into mature adipocytes upon appropriate stimulation [20,21]. This differentiation process can be divided into three phases (Fig. 2). First, preadipocytes are cultured in normal medium till reaching confluence. Second, the confluent adipocytes are cultured for 2-3 days in the presence of a hormonal cocktail containing dexamethasone, glucocorticoid and high dosage of insulin. This period is also called mitotic clonal expansion (MCE) because the preadipocytes re-enter the cell cycle and undergo another two rounds of cell division. Third, cells undergo terminal differentiation to become mature white adipocytes when cultured in the presence of media containing only insulin for another 3-10 days. During this period many genes involved in lipid uptake (e.g. LPL, CD36, FATP), lipid droplet formation (e.g. Tip47, PLIN), lipogenesis (e.g. FAS), glycerol uptake (e.g. AQP7) and glucose uptake (e.g. Glut4) are upregulated and lipid droplets appear.

PPARγ: an adipogenic transcription factor

The nuclear receptor Peroxisome Proliferator Activated Receptor γ (PPARγ) belongs to a family of lipid activated transcription factors involved in control of lipoprotein metabolism, insulin action and lipid and glucose homeostasis. There are 3 PPAR family members: PPARα, -β/δ and -γ. PPARα is dominant in mediating the effects of dietary fatty acids on gene expression in the liver [22]. Physiological experiments using PPARα knockout mice have shown that PPARα is especially important for the adaptive response to fasting [23]. PPARα is target of hypolipidemic fibrate drugs [22]. PPARδ is ubiquitously expressed and probably involved in fatty acid beta oxidation [24]. PPARδ-null mice model revealed its role in many different aspects of metabolic syndrome and developmental and homeostatic abnormalities like placental defects causing frequent embryonic lethality, decreased adipose mass, myelination defects, altered skin inflammatory responses and impaired wound healing. PPARγ is considered to be the master regulator of adipocyte differentiation and function for several reasons [25,26]. For example, *in vitro* differentiation of fibroblasts into mature adipocytes can be induced by introduction of PPARγ [27]. In addition, this protein regulates a large set of "adipocyte genes", involved in lipid and glucose metabolism, in a feed-forward loop with another transcription factor, C/EBPα [28,29]. Furthermore, PPARγ $^{-/-}$ mice are severely lipodystrophic, while PPARγ $^{+/-}$ mice have reduced amounts of adipose tissue [30,31,32,33]. PPARγ is also essential for the maintenance of adipose tissue, since conditional knock-out of the *Pparg* gene resulted in reduced *in vivo* survival of mature adipocytes [34]. Finally, human Familial partial lipodystrophy subtype 3 (FPLD3, MIM 604367) patients, harbouring heterozygous mutations in the *PPARG* gene, are characterized by aberrant fat distribution and metabolic disturbances, including insulin resistance and dyslipidemia [35].

There are two isoforms of the PPARγ protein: PPARγ1 and a longer isoform PPARγ2, which contains 28 extra N-terminal amino acids. PPARγ1 is expressed in multiple tissues (e.g. adipose tissue, liver, macrophages, lower intestine and skeletal muscle), but PPARγ2 is mainly expressed in adipose tissue. PPARγ forms heterodimers with retinoid X receptor (RXR) and binds to the PPAR response elements (PPREs) in enhancer sites of regulated genes. In the absence of ligand, nuclear receptor corepressors can bind to the heterodimer and recruit histone deacetylases (HDACs) to repress transcription. Ligand

activation induces a conformational change in the PPARγ protein, resulting in an exchange of corepressors for coactivator proteins and subsequent activation of target genes. In recent years, genome wide analysis of PPARγ binding sites has led to a new view on PPARγ-mediated gene regulation [28,29]. These findings indicate that PPARγ frequently act as a long-range enhancer or repressor of transcription, rather than a classical picture where PPARγ is primarily recruited to the proximal promoter.

Other adipogenic and anti-adipogenic transcription factors

While PPARγ may be considered the master regulator, many other transcription factors play important roles in adipogenesis, either in a pro- or anti-adipogenic manner (Fig. 2A and B). One of the earliest adipogenic transcription factors discovered is C/EBPα, which, together with its family members C/EBPβ and- δ, plays a positive role in adipogenesis [36]. These 3 factors belong to a family of highly conserved basic-leucine zipper proteins. C/EBPβ and –δ are induced early in adipogenesis; while C/EBPβ remains present and active in mature adipocytes, the expression of C/EBPδ is reduced in the differentiation process. C/EBPβ and –δ together induce PPARγ expression, and together they activate C/EBPα expression and in turn, C/EBPα can activate PPARγ expression [37]. Interestingly, ectopic PPARγ expression can completely rescue adipogenesis of C/EBPα deficient MEFs, suggesting that PPARγ is essential while C/EBPα is dispensable for adipogenesis [38]. The two factors do however seem to cooperate intensively in regulating the adipocyte gene program, as genome-wide analysis revealed that C/EBPα binding sites colocalize substantially with PPARγ [28,29]. In mouse models, the deletion of C/EBP genes results in severe abnormalities in fat tissue [39,40].

Another class of transcription factors implicated in adipogenesis are the Krüppel-like factors) (KLFs) [41]. The KLF family of zinc-finger transcription factors comprises 17 members that bind CACCC- and GC-rich DNA sequences and can act as repressors or activators, depending on the context. Eight KLF members have been implicated in adipogenesis. KLF4 works together with Krox20 in adipocyte differentiation and transactivate the C/EBPβ expression, thus KLF4 is recognized as an initiator of adipogenesis [42,43]. KLF6 represses Dlk1, an adipogenesis inhibitor, and thus has a positive effect on adipogenesis [44]. KLF5 directly activates the PPARγ2 promoter in early

adipogenesis [45]. KLF15 is also a positive regulator of adipogenesis [46], while KLF2, KLF3, KLF7 and KLF11 have repressive effects [47,48,49,50,51].
While the role of many transcription factors in adipogenesis is to induce the expression of a subsequent set of transcription factors, the role of some transcription factors is more complex [37]. One example is sterol regulatory element-binding transcription factor 1 (SREBP1), which regulates adipogenesis through at least 2 different mechanisms: it activates transcription of the *PPARG* gene [52], and it enhances production of endogenous PPARγ ligands [53]. Another example of a transcription factor with a complex role in adipogenesis is the nuclear receptor Rev-erbα, the mRNA expression of which is induced during adipogenesis [54]. While the increase in Rev-erbα is required for MCE, the protein needs to be subsequently degraded for continued differentiation [55].

Regulating the expression and/or activity of (anti) adipogenic transcription factors

The activity of (anti)adipogenic transcription factors is regulated by multiple pathways. As already described above, many (anti) adipogenic transcription factor directly regulate each others expression (Fig. 2B). Below we will discuss extracellular signaling and microRNAs, as well as the role of transcriptional co-factors in regulating the expression and/or activity of adipogenic transcription factors, focusing on PPARγ (Fig. 2B).

Extracellular signaling in the regulation of adipogenesis

Multiple extracellular signaling pathways have been implicated in adipogenesis [56]. Here we will briefly discuss the wingless-related MMTV integration site (WNT) pathway, the transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) pathways, and the TNFα pathway. Canonical WNT signaling promotes the differentiation of MSC into the osteogenic and myogenic lineage, while inhibiting the adipogenic lineage (reviewed in [57]). An important step in this process is the WNT-induced formation of a corepressor complex containing SETDB1, which inactivates ligand-bound PPARγ by histone methylation [58]. The TGF-β signalling pathway also prevents adipogenic fate in MSCs. TGF-β signalling activates small and mothers against decapentaplegic (Smad) proteins, transcriptional regulators that

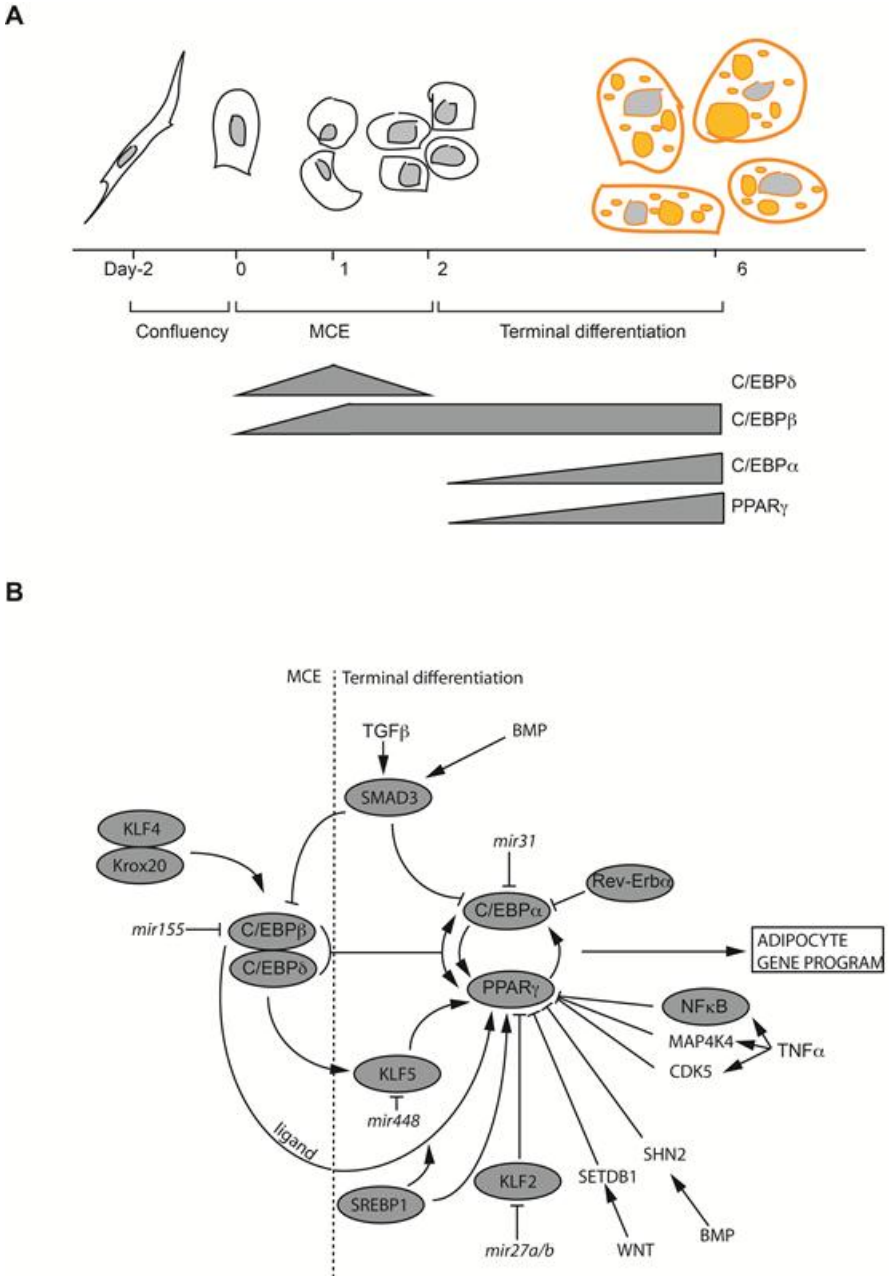

Figure 2. Schematic representation of adipogenesis. A, Indicated are the 3 stages of 3T3-L1 differentiation: growing to confluency, mitotic clonal expansion and terminal differentiation. B, Transcription factor cascades in adipogenesis. Selected transcription factors, extracellular signals and microRNAs are depicted. See text for details.

migrate to the nucleus and activate osteogenic and chondrogenic genes. SMAD3 binds to C/EBPs and inhibits their ability to activate the PPARγ promoter. Accordingly, RNAi- mediated repression of Smad3 in human adipose-tissue-derived mesenchymal stem cells (hASCs) promotes adipogenesis (Kim et al. 3093-102). BMPs have a more complex role in adipogenesis. BMPs activate Smad transcription factors same as TGF-β proteins. BMPs have been reported to regulate MSC determination depending on the concentration and type of the BMP involved. For instance, BMP-2 promotes adipogenesis in C3H10T1/2, a murine MSC cell line, at low concentrations, whereas favor osteogenic and chondrogenic phenotypes at higher concentrations (Wang et al. 57-71). The pro-adipogenic effects could be mediated by the BMP-signalling intermediate Schnurri-2 (SHN2), which may help to activate the PPARγ promoter [59].

The effects of TNFα on PPARγ expression and activity are of particular interest, as production of this cytokine by adipose tissue increases with obesity, which is viewed as an important step in local and systemic low grade inflammation observed in overweight and obese individuals [60,61]. The effects of TNFα on PPARγ are multifold. First, TNFα can activate NFκB signaling, which can transrepress PPARγ-mediated gene transcription [62]. Second, TNFα-mediated activation of MAP4K4 has been shown to destabilize PPARγ mRNA [63,64]. Finally, TNFα can activate cyclin-dependent kinase 5 (CDK5), a kinase that can selectively repress PPARγ activity by phosphorylating serine 273 [65].

MicroRNAs in the regulation of adipogenesis

Besides being controlled by various proteins, the expression of adipogenic transcription factors is also regulated by microRNAs (miRNAs). miRNAs are a class of small non-coding RNAs (approximately 22 nucleotides in length) that are increasingly being recognized as viable therapeutic targets for a serious of diseases. miRNAs can be transcribed by either RNA polymerase II or III into primary transcripts and these transcripts are cleaved in the nucleus by the enzyme Drosha and its cofactor Pasha at the bottom of their stem loops to make ~ 70 nucleotide precursors called pre-miRNA. Afterwards, premiRNAs are exported to the cytosol and there they are processed by other enzymes such as Dicer to generate an approximately 22 nucleotide mature miRNA duplex that can be incorporated into an RNA-induced silencing complex (RISC) to be active

as a post-transcriptional regulator. miRNA is involved for the terminal differentiation of adipocytes coming from the evidence that knockout of Dicer, which is an essential enzyme for miRNA maturation in preadipocytes before induction dramatically impaired lipogenesis and downregulated several fold adipocyte markers such as PPARγ, FAS, GLUT4, and FABP4. Adipose conditional Dicer knockout mice displayed a severe depletion of adipose tissue [66]. During adipogenesis, around 8.8% miRNA was significantly changed between non-induced and induced cells, which proved an important role of miRNA in adipocyte development. Several individual miRNA has been shown to be important for adipogenesis as well. For example, let-7 regulates adipogenesis by negatively regulating the clonal expansion of preadipocytes. The miR-17-92 cluster, which could promotes cell proliferation in several cell lines, targets tumor suppressor retinoblastoma2 (Rb2)/p130 mRNA and accelerates adipogenesis when ectopically expressed in 3T3-L1 cells. miRNAs that target the other transcriptional factors in adipogenesis is regulating adipogenesis. miR-27a and miR-27b are negative regulators of adipogenesis, and both have been shown to directly target PPARγ mRNA [67,68,69]. miR-31 directly targets C/EBPα, and levels of this miRNA are downregulated [70]. In the context of macrophage studies, miR-155 directly targets C/EBPβ [71]. miR-143 targets ERK5 and thus accelerates adipogenesis in 3T3-L1 cells [72] presumably by preventing the phosphorylation and inactivation of C/EBPβ. Additionally, miR-143 enrichment in mature murine adipose tissue is several folds higher than in 3T3-L1 adipocytes [73]. Also, miR-448 negatively regulates adipogenesis by targeting Klf5 [74].

Transcriptional cofactors for PPARγ

A number of transcriptional cofactors, non-DNA binding proteins that can activate or repress transcription (coactivators and corepressors, respectively), have been implicated in the adipogenic functions of PPARγ [75,76]. In the absence of ligand, nuclear receptor corepressors like nuclear receptor corepressor protein (NCoR) [77] and silencing mediator of retinoid and thyroid hormone receptors (SMRT) [78] can bind to PPARγ and recruit histone deacetylases (HDACs) to repress transcription [79,80]. Ligand binding alters PPARγ's affinity for a number of coactivators, which are involved in chromatin remodeling by histone modification and nucleosome mobilization, leading to the

recruitment of the basal transcription machinery to PPAR target genes [81,82]. A short protein sequence called LXXLL motif is necessary for many coactivators to bind to nuclear receptors, where L is leucine and X is any amino acid [83]. Amongst others, PPARγ coactivators include SRC family members, SWI/SNF chromatin remodeling complex, and the mediator complex (also referred to as thyroid receptor associated protein (TRAP)/vitamin D receptor interacting proteins (DRIP) complex). SRC belongs to the p160 family. These proteins have weak histone acetyltransferase (HAT) activities while their main function probably lies in providing foundations upon which coactivator complexes are assembled [84]. The coactivators they recruit include cAMP responsive element binding protein (CREB) binding protein (CBP)/p300. CBP/p300 complex possesses HAT activity and aids in remodeling chromatin to allow transcriptional activation [82]. The SWI/SNF complex is thought to be targeted to nuclear receptors by interaction with receptors, coactivators or general transcription machinery [82]. This complex also functions in PPARγ-mediated transcription [85,86,87]. The Mediator complex also contributes to PPARγ-mediated transcription by bridging this transcription factor to the basal transcription machinery [88,89,90]. More recently, the acetyltransferase HIV-1 Tat interacting protein 60 (Tip60) was identified as an essential cofactor in adipogenesis [91]. This protein will be discussed in more detail below.
The role of some of these cofactors in adipocyte differentiation and/or function has been studied *in vivo* (e.g. SRC1 and -2; [92], RIP140 [93]), but, whole body gene knock outs of several cofactor genes results in embryonic lethality (e.g. NCoR[94]; SMRT[95]; CBP and p300 [96]). Alternative approaches have therefore been used, including tissue-specific homozygous gene inactivation (e.g. [97]), or generation of animals bearing specific knockin mutations [98,99,100,101].

1.2 The transcriptional cofactor and acetyltransferase Tip60

The transcriptional cofactor and acetyltransferase Tip60 (also referred to as K (lysine) acetyltransferase 5; KAT5) was originally discovered by two independent approaches. It was identified as an HIV-Tat interacting protein that could modestly activate Tat-dependent transcription [102]. In addition, Tip60 (isoform 3) was isolated as a cPLA2 interacting protein (and named PLA2 interacting protein, PLIP) in a yeast two-hybrid screen [103]. Tip60 is part of a small family of MYST

acetyltransferases, named after its founding members MOZ, Ybf2/Sas3 (yeast), Sas2 (yeast) and Tip60, which share a highly conserved MYST acetyltransferase domain, but display limited homology outside this region [104,105]. The mammalian MYST family encompasses Tip60, MOZ, MORF, HBO1 (Fig. 3). Tip60 can acetylate both histones and non-histone proteins [104,106]. Tip60 is highly conserved between species and be found from humans to Drosophila and C.elegans, while yeast contains the Esa1 homologue. In mammals, 3 Tip60 isoforms have been reported: a long isoform 1 (aa 1-546 aa), isoform 2 (aa 34-546) and isoform 3, which has an internal deletion (Δ128-180). Besides the centrally located MYST acetyltransferase domain, several other domains can be distinguished in the Tip60 protein. Tip60 contains an N-terminal chromodomain, which can recognize modified histones (H3K4me2/3, H3K9me2/3, H3K27me2/3) and/or DNA [107]. A C2HC type zinc finger, which may be involved in substrate binding, is located within the MYST acetyltransferase domain [108]. Finally, Tip60 harbours an LXXLL motif in its C-terminus, which can bind nuclear receptors ([109]; see also below). Tip60 is part of a large multi-protein complex [110,111,112], implicated in many fundamental cellular processes like transcription, DNA damage repair, cell cycle control and apoptosis [106,113]. In agreement with this, Tip60 knock-out mice display embryonic lethality [114,115] and Tip60 depletion in D. melanogaster results in early pupal lethality [116].

We have recently identified Tip60 as a PPARγ interacting protein [91]. Chromatin immunoprecipitation experiments showed that the endogenous Tip60 protein is recruited to PPARγ target genes in mature 3T3-L1 adipocytes, but not in pre-adipocytes, indicating that Tip60 recruitment critically depends on PPARγ. Importantly, we show that in common with disruption of PPARγ function, siRNA-mediated reduction of Tip60 protein impairs differentiation of 3T3-L1 pre-adipocytes. Taken together, these findings qualify the acetyltransferase Tip60 as a novel adipogenic factor [91].

Tip60 has previously been implicated in signalling through several other nuclear receptors, including the androgen receptor (AR), glucocorticoid receptor (GR), estrogen receptor (ER) and RORα [109,117,118,119]. In the case of AR, GR and ER, Tip60 was reported to interact directly with these class I nuclear receptors through their LBDs and the interactions required the presence of the respective ligands as well as an intact LXXLL motif in the Tip60 protein [109]. The failure to

detect interactions between Tip60 and the LBDs of several class II nuclear receptors (Thyroid hormone receptor (TR), vitamin D receptor (VDR) and retinoid X receptor (RXR)), together with the lack of upregulation of these receptors by Tip60 led to the hypothesis that Tip60 was a coactivator specific for class I nuclear receptors [109]. We have recently shown that Tip60 can potentiate the activity of the class I nuclear receptor PPARγ, but this occurs through an alternative molecular mechanism [91]. In contrast to the Tip60-class I NR interactions, the Tip60-PPARγ interaction can occur in the absence of ligand and is independent of the LBD-cofactor (LXXLL) interaction interface. Furthermore, using chimeric NRs and *in vitro* protein-protein interaction assays, we demonstrated that Tip60 targets the N-terminal AF1 region of PPARγ, a domain involved in isotype-specific gene expression and adipogenesis [120,121]. We concluded therefore that the same coactivator (or coactivator complexes) can play a role in the activation process of closely related transcription factors through distinct molecular mechanisms.

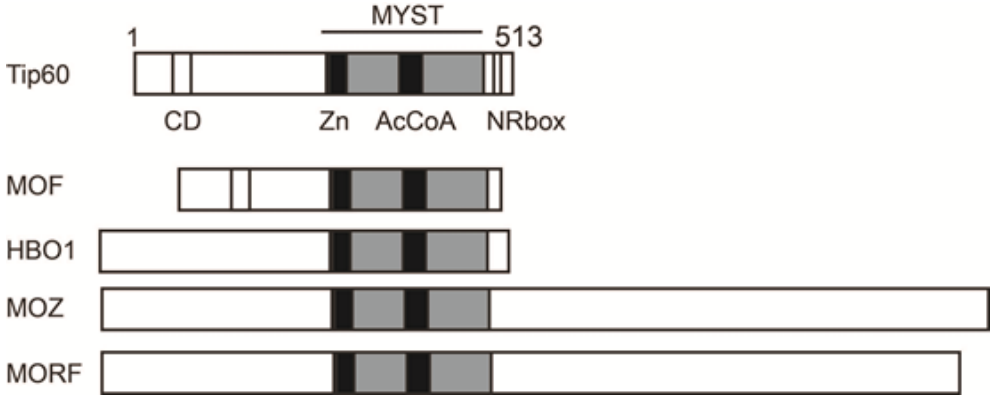

Figure 3. Schematic representation of the mammalian MYST family of acetyltransferases. For Tip60, isoform 2 is depicted. Indicated are the enzymatic MYST domain with its characteristic zinc finger (Zn), the acetylCoA binding site (AcCoA), the chromodomain (CD) and the nuclear receptor interaction box (NR box; LXXL motif).

1.3 Aim of this thesis

Since adipose tissue is increasingly being recognized as a key regulator of whole-body energy homeostasis and consequently as a prime therapeutic target for metabolic syndrome, adipocyte differentiation

and biology are under intensive study. There are more and more factors being discovered to be relevant for adipogenesis. Increasing evidence shows that adipogenesis is a hierarchical sequence of molecular events: different factors are regulated at different time points, ultimately leading to increased expression and activity of the master regulator of adipogenesis, PPARγ. A detailed understanding of the chronological steps in adipogenesis is therefore essential to understand the role of adipocytes in energy metabolism and obesity-related human health problems like type 2 diabetes. In this thesis we investigated the role of the transcriptional co-factor Tat-interacting protein 60 (Tip60) in adipogenesis.

References

1. Rosen ED, Spiegelman BM (2006) Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444: 847-853.
2. Guilherme A, Virbasius JV, Puri V, Czech MP (2008) Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. NatRevMolCell Biol 9: 367-377.
3. Tilg H, Moschen AR (2006) Adipocytokines: mediators linking adipose tissue, inflammation and immunity. NatRevImmunol 6: 772-783.
4. Cannon B, Nedergaard J (2004) Brown adipose tissue: function and physiological significance. Physiological reviews 84: 277-359.
5. Lean ME, James WP, Jennings G, Trayhurn P (1986) Brown adipose tissue uncoupling protein content in human infants, children and adults. Clinical science 71: 291-297.
6. Frontini A, Cinti S (2010) Distribution and development of brown adipocytes in the murine and human adipose organ. Cell metabolism 11: 253-256.
7. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, et al. (2009) Identification and importance of brown adipose tissue in adult humans. The New England journal of medicine 360: 1509-1517.
8. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, et al. (2009) Cold-activated brown adipose tissue in healthy men. The New England journal of medicine 360: 1500-1508.
9. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, et al. (2009) Functional brown adipose tissue in healthy adults. The New England journal of medicine 360: 1518-1525.
10. Park KW, Halperin DS, Tontonoz P (2008) Before they were fat: adipocyte progenitors. Cell metabolism 8: 454-457.
11. Seale P, Bjork B, Yang W, Kajimura S, Chin S, et al. (2008) PRDM16 controls a brown fat/skeletal muscle switch. Nature 454: 961-967.
12. Guerra C, Koza RA, Yamashita H, Walsh K, Kozak LP (1998) Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. The Journal of clinical investigation 102: 412-420.
13. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, et al. (2012) Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell 150: 366-376.

14. Cinti S (2011) Between brown and white: novel aspects of adipocyte differentiation. Annals of medicine 43: 104-115.
15. Qatanani M, Lazar MA (2007) Mechanisms of obesity-associated insulin resistance: many choices on the menu. Genes Dev 21: 1443-1455.
16. Kahn SE, Hull RL, Utzschneider KM (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444: 840-846.
17. Van Gaal LF, Mertens IL, De Block CE (2006) Mechanisms linking obesity with cardiovascular disease. Nature 444: 875-880.
18. Rasouli N, Kern PA (2008) Adipocytokines and the metabolic complications of obesity. JClinEndocrinolMetab 93: S64-S73.
19. Poulos SP, Dodson MV, Hausman GJ (2010) Cell line models for differentiation: preadipocytes and adipocytes. ExpBiolMed (Maywood) 235: 1185-1193.
20. Green H, Kehinde O (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5: 19-27.
21. Green H, Kehinde O (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7: 105-113.
22. Lefebvre P, Chinetti G, Fruchart JC, Staels B (2006) Sorting out the roles of PPAR alpha in energy metabolism and vascular horneostasis. JClinInvest 116: 571-580.
23. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, et al. (1999) Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. JClinInvest 103: 1489-1498.
24. Barish GD, Narkar VA, Evans RM (2006) PPAR delta: a dagger in the heart of the metabolic syndrome. JClinInvest 116: 590-597.
25. Tontonoz P, Spiegelman BM (2008) Fat and beyond: the diverse biology of PPARgamma. AnnuRevBiochem 77: 289-312.
26. Lehrke M, Lazar MA (2005) The many faces of PPARgamma. Cell 123: 993-999.
27. Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79: 1147-1156.
28. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, et al. (2008) Genome-wide profiling of PPAR{gamma}:RXR and RNA polymerase II occupancy reveals temporal activation of distinct

metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev 22: 2953-2967.
29. Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, et al. (2008) PPAR{gamma} and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes Dev 22: 2941-2952.
30. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, et al. (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. MolCell 4: 585-595.
31. Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, et al. (1999) PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. MolCell 4: 597-609.
32. Nadra K, Quignodon L, Sardella C, Joye E, Mucciolo A, et al. (2010) PPARgamma in placental angiogenesis. Endocrinology 151: 4969-4981.
33. Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, et al. (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. MolCell 4: 611-617.
34. Imai T, Takakuwa R, Marchand S, Dentz E, Bornert JM, et al. (2004) Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. ProcNatlAcadSciUSA 101: 4543-4547.
35. Hegele RA (2005) Lessons from human mutations in PPARgamma. IntJObes(Lond) 29 Suppl 1:S31-5.: S31-S35.
36. Yeh WC, Cao Z, Classon M, McKnight SL (1995) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes & development 9: 168-181.
37. Farmer SR (2006) Transcriptional control of adipocyte formation. Cell Metab 4: 263-273.
38. Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, et al. (2002) C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 16: 22-26.
39. Tanaka T, Yoshida N, Kishimoto T, Akira S (1997) Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. The EMBO journal 16: 7432-7443.
40. Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, et al. (1995) Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269: 1108-1112.
41. Brey CW, Nelder MP, Hailemariam T, Gaugler R, Hashmi S (2009) Kruppel-like family of transcription factors: an emerging new

frontier in fat biology. International journal of biological sciences 5: 622-636.
42. Birsoy K, Chen Z, Friedman J (2008) Transcriptional regulation of adipogenesis by KLF4. Cell Metab 7: 339-347.
43. Chen Z, Torrens JI, Anand A, Spiegelman BM, Friedman JM (2005) Krox20 stimulates adipogenesis via C/EBPbeta-dependent and -independent mechanisms. Cell metabolism 1: 93-106.
44. Li D, Yea S, Li S, Chen Z, Narla G, et al. (2005) Kruppel-like factor-6 promotes preadipocyte differentiation through histone deacetylase 3-dependent repression of DLK1. The Journal of biological chemistry 280: 26941-26952.
45. Oishi Y, Manabe I, Tobe K, Tsushima K, Shindo T, et al. (2005) Kruppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. Cell Metab 1: 27-39.
46. Mori T, Sakaue H, Iguchi H, Gomi H, Okada Y, et al. (2005) Role of Kruppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis. The Journal of biological chemistry 280: 12867-12875.
47. Sue N, Jack BH, Eaton SA, Pearson RC, Funnell AP, et al. (2008) Targeted disruption of the basic Kruppel-like factor gene (Klf3) reveals a role in adipogenesis. Molecular and cellular biology 28: 3967-3978.
48. Banerjee SS, Feinberg MW, Watanabe M, Gray S, Haspel RL, et al. (2003) The Kruppel-like factor KLF2 inhibits peroxisome proliferator-activated receptor-gamma expression and adipogenesis. The Journal of biological chemistry 278: 2581-2584.
49. Kawamura Y, Tanaka Y, Kawamori R, Maeda S (2006) Overexpression of Kruppel-like factor 7 regulates adipocytokine gene expressions in human adipocytes and inhibits glucose-induced insulin secretion in pancreatic beta-cell line. Molecular endocrinology 20: 844-856.
50. Cao S, Fernandez-Zapico ME, Jin D, Puri V, Cook TA, et al. (2005) KLF11-mediated repression antagonizes Sp1/sterol-responsive element-binding protein-induced transcriptional activation of caveolin-1 in response to cholesterol signaling. The Journal of biological chemistry 280: 1901-1910.
51. Neve B, Fernandez-Zapico ME, Ashkenazi-Katalan V, Dina C, Hamid YH, et al. (2005) Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. Proceedings

of the National Academy of Sciences of the United States of America 102: 4807-4812.
52. Fajas L, Schoonjans K, Gelman L, Kim JB, Najib J, et al. (1999) Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism. MolCell Biol 19: 5495-5503.
53. Kim JB, Wright HM, Wright M, Spiegelman BM (1998) ADD1/SREBP1 activates PPARgamma through the production of endogenous ligand. ProcNatlAcadSciUSA 95: 4333-4337.
54. Chawla A, Lazar MA (1993) Induction of Rev-ErbA alpha, an orphan receptor encoded on the opposite strand of the alpha-thyroid hormone receptor gene, during adipocyte differentiation. The Journal of biological chemistry 268: 16265-16269.
55. Wang J, Lazar MA (2008) Bifunctional role of Rev-erbalpha in adipocyte differentiation. Molecular and cellular biology 28: 2213-2220.
56. Rosen ED, MacDougald OA (2006) Adipocyte differentiation from the inside out. NatRevMolCell Biol 7: 885-896.
57. Christodoulides C, Lagathu C, Sethi JK, Vidal-Puig A (2009) Adipogenesis and WNT signalling. Trends in endocrinology and metabolism: TEM 20: 16-24.
58. Takada I, Mihara M, Suzawa M, Ohtake F, Kobayashi S, et al. (2007) A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nature cell biology 9: 1273-1285.
59. Jin W, Takagi T, Kanesashi SN, Kurahashi T, Nomura T, et al. (2006) Schnurri-2 controls BMP-dependent adipogenesis via interaction with Smad proteins. Developmental cell 10: 461-471.
60. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM (1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. JClinInvest 95: 2409-2415.
61. Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259: 87-91.
62. Suzawa M, Takada I, Yanagisawa J, Ohtake F, Ogawa S, et al. (2003) Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. NatCell Biol 5: 224-230.

63. Tang XQ, Guilherme A, Chakladar A, Powelka AM, Konda S, et al. (2006) An RNA interference-based screen identifies MAP4K4/NIK as a negative regulator of PPAR gamma, adipogenesis, and insulin-responsive hexose transport. ProcNatlAcadSciUSA 103: 2087-2092.
64. Guntur KV, Guilherme A, Xue L, Chawla A, Czech MP (2010) Map4k4 negatively regulates peroxisome proliferator-activated receptor (PPAR) gamma protein translation by suppressing the mammalian target of rapamycin (mTOR) signaling pathway in cultured adipocytes. The Journal of biological chemistry 285: 6595-6603.
65. Choi JH, Banks AS, Estall JL, Kajimura S, Bostrom P, et al. (2010) Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5. Nature 466: 451-456.
66. Mudhasani R, Imbalzano AN, Jones SN (2010) An essential role for Dicer in adipocyte differentiation. Journal of cellular biochemistry 110: 812-816.
67. Lin Q, Gao Z, Alarcon RM, Ye J, Yun Z (2009) A role of miR-27 in the regulation of adipogenesis. The FEBS journal 276: 2348-2358.
68. Karbiener M, Fischer C, Nowitsch S, Opriessnig P, Papak C, et al. (2009) microRNA miR-27b impairs human adipocyte differentiation and targets PPARgamma. Biochemical and biophysical research communications 390: 247-251.
69. Kim SY, Kim AY, Lee HW, Son YH, Lee GY, et al. (2010) miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARgamma expression. Biochemical and biophysical research communications 392: 323-328.
70. Tang YF, Zhang Y, Li XY, Li C, Tian W, et al. (2009) Expression of miR-31, miR-125b-5p, and miR-326 in the adipogenic differentiation process of adipose-derived stem cells. Omics : a journal of integrative biology 13: 331-336.
71. Worm J, Stenvang J, Petri A, Frederiksen KS, Obad S, et al. (2009) Silencing of microRNA-155 in mice during acute inflammatory response leads to derepression of c/ebp Beta and down-regulation of G-CSF. Nucleic acids research 37: 5784-5792.
72. Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, et al. (2004) MicroRNA-143 regulates adipocyte differentiation. The Journal of biological chemistry 279: 52361-52365.
73. Xie H, Lim B, Lodish HF (2009) MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. Diabetes 58: 1050-1057.

74. Kinoshita M, Ono K, Horie T, Nagao K, Nishi H, et al. (2010) Regulation of adipocyte differentiation by activation of serotonin (5-HT) receptors 5-HT2AR and 5-HT2CR and involvement of microRNA-448-mediated repression of KLF5. Molecular endocrinology 24: 1978-1987.
75. Feige JN, Auwerx J (2007) Transcriptional coregulators in the control of energy homeostasis. Trends Cell Biol 17: 292-301.
76. Koppen A, Kalkhoven E (2010) Brown vs white adipocytes: the PPARgamma coregulator story. FEBS Lett 584: 3250-3259.
77. Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, et al. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377: 397-404.
78. Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377: 454-457.
79. Yu C, Markan K, Temple KA, Deplewski D, Brady MJ, et al. (2005) The nuclear receptor corepressors NCoR and SMRT decrease peroxisome proliferator-activated receptor gamma transcriptional activity and repress 3T3-L1 adipogenesis. JBiolChem 280: 13600-13605.
80. Stanley TB, Leesnitzer LM, Montana VG, Galardi CM, Lambert MH, et al. (2003) Subtype specific effects of peroxisome proliferator-activated receptor ligands on corepressor affinity. Biochemistry 42: 9278-9287.
81. Hermanson O, Glass CK, Rosenfeld MG (2002) Nuclear receptor coregulators: multiple modes of modification. Trends in endocrinology and metabolism: TEM 13: 55-60.
82. Glass CK, Rosenfeld MG (2000) The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 14: 121-141.
83. Heery DM, Kalkhoven E, Hoare S, Parker MG (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors [see comments]. Nature 387: 733-736.
84. Leo C, Chen JD (2000) The SRC family of nuclear receptor coactivators. Gene 245: 1-11.
85. Salma N, Xiao H, Mueller E, Imbalzano AN (2004) Temporal recruitment of transcription factors and SWI/SNF chromatin-remodeling enzymes during adipogenic induction of the peroxisome proliferator-activated receptor gamma nuclear hormone receptor. Molecular and cellular biology 24: 4651-4663.

86. Debril MB, Gelman L, Fayard E, Annicotte JS, Rocchi S, et al. (2004) Transcription factors and nuclear receptors interact with the SWI/SNF complex through the BAF60c subunit. The Journal of biological chemistry 279: 16677-16686.
87. Caramel J, Medjkane S, Quignon F, Delattre O (2008) The requirement for SNF5/INI1 in adipocyte differentiation highlights new features of malignant rhabdoid tumors. Oncogene 27: 2035-2044.
88. Ge K, Guermah M, Yuan CX, Ito M, Wallberg AE, et al. (2002) Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis. Nature 417: 563-567.
89. Ge K, Cho YW, Guo H, Hong TB, Guermah M, et al. (2008) Alternative mechanisms by which mediator subunit MED1/TRAP220 regulates peroxisome proliferator-activated receptor gamma-stimulated adipogenesis and target gene expression. Molecular and cellular biology 28: 1081-1091.
90. Grontved L, Madsen MS, Boergesen M, Roeder RG, Mandrup S (2010) MED14 tethers mediator to the N-terminal domain of peroxisome proliferator-activated receptor gamma and is required for full transcriptional activity and adipogenesis. Molecular and cellular biology 30: 2155-2169.
91. van Beekum O, Brenkman AB, Grontved L, Hamers N, van den Broek NJ, et al. (2008) The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARgamma. Endocrinology 149: 1840-1849.
92. Picard F, Gehin M, Annicotte J, Rocchi S, Champy MF, et al. (2002) SRC-1 and TIF2 control energy balance between white and brown adipose tissues. Cell 111: 931-941.
93. Leonardsson G, Steel JH, Christian M, Pocock V, Milligan S, et al. (2004) Nuclear receptor corepressor RIP140 regulates fat accumulation. Proceedings of the National Academy of Sciences of the United States of America 101: 8437-8442.
94. Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, et al. (2000) Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell 102: 753-763.
95. Jepsen K, Solum D, Zhou T, McEvilly RJ, Kim HJ, et al. (2007) SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. Nature 450: 415-419.

96. Yao TP, Oh SP, Fuchs M, Zhou ND, Ch'ng LE, et al. (1998) Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell 93: 361-372.
97. Li P, Fan W, Xu J, Lu M, Yamamoto H, et al. (2011) Adipocyte NCoR knockout decreases PPARgamma phosphorylation and enhances PPARgamma activity and insulin sensitivity. Cell 147: 815-826.
98. Nofsinger RR, Li P, Hong SH, Jonker JW, Barish GD, et al. (2008) SMRT repression of nuclear receptors controls the adipogenic set point and metabolic homeostasis. Proceedings of the National Academy of Sciences of the United States of America 105: 20021-20026.
99. Shikama N, Lutz W, Kretzschmar R, Sauter N, Roth JF, et al. (2003) Essential function of p300 acetyltransferase activity in heart, lung and small intestine formation. EMBO J 22: 5175-5185.
100. Yamamoto H, Williams EG, Mouchiroud L, Canto C, Fan W, et al. (2011) NCoR1 is a conserved physiological modulator of muscle mass and oxidative function. Cell 147: 827-839.
101. Roth JF, Shikama N, Henzen C, Desbaillets I, Lutz W, et al. (2003) Differential role of p300 and CBP acetyltransferase during myogenesis: p300 acts upstream of MyoD and Myf5. EMBO J 22: 5186-5196.
102. Kamine J, Elangovan B, Subramanian T, Coleman D, Chinnadurai G (1996) Identification of a cellular protein that specifically interacts with the essential cysteine region of the HIV-1 Tat transactivator. Virology 216: 357-366.
103. Sheridan AM, Force T, Yoon HJ, O'Leary E, Choukroun G, et al. (2001) PLIP, a novel splice variant of Tip60, interacts with group IV cytosolic phospholipase A(2), induces apoptosis, and potentiates prostaglandin production. Molecular and cellular biology 21: 4470-4481.
104. Yang XJ (2004) The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. Nucleic Acids Res 32: 959-976.
105. Utley RT, Cote J (2003) The MYST family of histone acetyltransferases. CurrTopMicrobiolImmunol 274: 203-236.
106. Sapountzi V, Logan IR, Robson CN (2006) Cellular functions of TIP60. IntJBiochemCell Biol 38: 1496-1509.

107. Ruthenburg AJ, Li H, Patel DJ, Allis CD (2007) Multivalent engagement of chromatin modifications by linked binding modules. NatRevMolCell Biol 8: 983-994.
108. Akhtar A, Becker PB (2001) The histone H4 acetyltransferase MOF uses a C2HC zinc finger for substrate recognition. EMBO Rep 2: 113-118.
109. Gaughan L, Brady ME, Cook S, Neal DE, Robson CN (2001) Tip60 is a co-activator specific for class I nuclear hormone receptors. JBiolChem 276: 46841-46848.
110. Doyon Y, Cayrou C, Ullah M, Landry AJ, Cote V, et al. (2006) ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. MolCell 21: 51-64.
111. Doyon Y, Cote J (2004) The highly conserved and multifunctional NuA4 HAT complex. CurrOpinGenetDev 14: 147-154.
112. Ikura T, Ogryzko VV, Grigoriev M, Groisman R, Wang J, et al. (2000) Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell 102: 463-473.
113. Squatrito M, Gorrini C, Amati B (2006) Tip60 in DNA damage response and growth control: many tricks in one HAT. Trends Cell Biol 16: 433-442.
114. Hu Y, Fisher JB, Koprowski S, McAllister D, Kim MS, et al. (2009) Homozygous disruption of the Tip60 gene causes early embryonic lethality. DevDyn 238: 2912-2921.
115. Gorrini C, Squatrito M, Luise C, Syed N, Perna D, et al. (2007) Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature 448: 1063-1067.
116. Zhu X, Singh N, Donnelly C, Boimel P, Elefant F (2007) The cloning and characterization of the histone acetyltransferase human homolog Dmel\TIP60 in Drosophila melanogaster: Dmel\TIP60 is essential for multicellular development. Genetics 175: 1229-1240.
117. Gaughan L, Logan IR, Cook S, Neal DE, Robson CN (2002) Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. JBiolChem 277: 25904-25913.
118. Gold DA, Baek SH, Schork NJ, Rose DW, Larsen DD, et al. (2003) RORalpha coordinates reciprocal signaling in cerebellar development through sonic hedgehog and calcium-dependent pathways. Neuron 40: 1119-1131.

119. Metivier R, Penot G, Hubner MR, Reid G, Brand H, et al. (2003) Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115: 751-763.
120. Hummasti S, Tontonoz P (2006) The peroxisome proliferator-activated receptor N-terminal domain controls isotype-selective gene expression and adipogenesis. MolEndocrinol 20: 1261-1275.
121. Bugge A, Grontved L, Aagaard MM, Borup R, Mandrup S (2009) The PPARgamma2 A/B-domain plays a gene-specific role in transactivation and cofactor recruitment. Molecular endocrinology 23: 794-808.

Chapter 2

EARLY ADIPOGENESIS IS REGULATED THROUGH USP7-MEDIATED DEUBIQUITINATION OF THE HISTONE ACETYLTRANSFERASE TIP60

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SUMMARY
Transcriptional coregulators, such as the acetyltransferase Tip60, play key roles in complex cellular processes such as differentiation. While posttranslational modifications have emerged as an important mechanism to regulate transcriptional coregulator activity, the identification of the corresponding demodyfying enzymes has remained elusive. Here we show that the expression of the Tip60 protein, which is essential for adipocyte differentiation, is regulated through polyubiquitination on multiple residues. USP7, a dominant deubiquitinating enzyme (DUB) in 3T3-L1 adipocytes and mouse adipose tissue, deubiquitinated Tip60 both in intact cells and *in vitro*, and increased Tip60 protein levels. Inhibition of USP7 expression and activity decreased adipogenesis. Transcriptome analysis revealed several cell cycle genes to be co-regulated by both Tip60 and USP7. Knock down of either factor resulted in impaired mitotic clonal expansion, an early step in adipogenesis. These results therefore reveal deubiquitination of a transcriptional coregulator to be a key mechanism in the regulation of early adipogenesis.

INTRODUCTION

Adipogenesis, the differentiation of fibroblast-like mesenchymal stem cells into adipocytes, is studied extensively for its importance in the regulation of energy metabolism. A widely accepted adipogenesis model is the 3T3-L1 cell line, which can efficiently be differentiated into mature adipocytes by hormonal stimulation under experimental conditions [1,2]. During adipogenesis cells are first growth-arrested, subsequently treated with differentiation inducers to synchronously re-enter the cell cycle and undergo mitotic clonal expansion (MCE), and finally exit the cell cycle to undergo terminal differentiation. MCE is a prerequisite for 3T3-L1 differentiation [3], underscoring the intimate relationship between cell cycle regulation and differentiation in this model system for adipogenesis.

Adipogenesis is regulated by a cascade of transcription factors, including the CCAT enhancer binding proteins (C/EBPs) and Peroxisome proliferator activator receptor (PPAR) [4,5]. Post-translational modifications (PTM), such as phosphorylation, acetylation, sumoylation and ubiquitination, can regulate the transcriptional output of these transcription factors (reviewed in [6,7]). PTM can affect various aspects of transcription factor function including subcellular localization, protein stability and interactions with transcriptional coregulators. Interestingly, several transcriptional co-regulators are also subject to PTM, offering the possibility to fine-tune transcriptional activity [8]. A clear example of this is the coactivator SRC-3, which is essential for adipogenesis *in vitro* and *in vivo* [9-11]. SRC-3 is subject to phosphorylation, methylation, acetylation and ubiquitination, regulating its subcellular localization, activity and stability (reviewed in [8,12]). Another example of a coregulator undergoing PTM is Tip60 (HIV-1 Tat interacting protein 60), a member of the MYST family of acetyltransferases [13,14]. Tip60 is the catalytic subunit of the highly conserved NuA4 acetyltransferase complex [15,16], which plays a key role in transcription regulation and DNA damage repair. Tip60 acetylates histone proteins (H4, H2A, H2A.X and H2A.Z) and various transcription factors, thereby activating or repressing transcription [17]. The Tip60 complex can also modify chromatin around DNA double-strand breaks to support repair, demonstrating an additional important cellular function [18]. Its role in these two fundamental cellular processes may explain the embryonic lethality observed in Tip60 knock-out mice [19,20] and early pupal lethality after Tip60 depletion in *D. melanogaster* [21].

Similar to SRC3, Tip60 can undergo multiple PTM, including phosphorylation [22], sumoylation [23], and ubiquitination [24]. Phosphorylation and sumoylation regulate the acetyltransferase activity of Tip60 [22,23], and ubiquitination results in degradation of the protein by the proteasome [24]. While PTM therefore present an important mechanism to regulate the activity of transcriptional coregulators such as SRC-3 and Tip60, demodification may play an equally important role in balancing the transcriptional output. However, to the best of our knowledge no enzymes have been identified so far which can, for example, deubiquitinate coregulator proteins. Recently, we found Tip60 to be essential for the differentiation of 3T3-L1 adipocytes [25]. Since Tip60 protein levels, but not mRNA levels, are upregulated during adipogenesis [25], and since Tip60 can be degraded by the proteasome [24,26], these findings suggest that the Tip60 protein may be stabilized through deubiquitination during adipogenesis. Here we describe the identification of USP7 (also known as HAUSP) as an essential deubiquitinase in adipogenesis. Tip60 can be deubiquitinated by USP7 in intact cells and *in vitro*, qualifying Tip60 as a direct substrate of USP7. RNAi-mediated knock down of USP7 and Tip60 affects a common set of target genes, which are predominantly involved in cell cycle regulation. In agreement with this, RNAi-mediated knock down of USP7 and Tip60 impaired MCE. Taken together, our findings reveal deubiquitination of a transcriptional coregulator as a novel mechanism in the regulation of early adipogenesis.

RESULTS

The deubiquitinase USP7 is essential for adipogenesis

Expression of the Tip60 protein, but not mRNA, increases during the first stages of 3T3-L1 differentiation (Fig. 1a; [25]), suggesting that this protein may be regulated by deubiquitination during adipogenesis. To identify specific DUB activities during adipogenesis, we made use of an HA-tagged probe that covalently binds active DUBs [27,28]. Incubation of 3T3-L1 lysates with this probe followed by Western blotting with an HA antibody revealed only two major DUB activities during adipogenesis (Fig. 1b), while 293T cells (Fig. 1b) and other cells types [27] display multiple activities. Interestingly, the increase in enzymatic activity of the two dominant DUBs during adipogenesis coincided with increased Tip60 protein expression (day 2; Fig. 1a and 1b). Based on the molecular weights (~130 and ~37 kD) we predicted that the high molecular weight activity may present USP7 (also known as HAUSP), a DUB best known for its involvement in the p53-mdm2 pathway [29,30]. Immunoprecipitation of active DUBs through the HA-tagged probe from mature 3T3-L1 adipocytes followed by Western blotting with anti-USP7 antibodies showed that the high molecular weight DUB activity in mature adipocytes was indeed USP7 (Fig. 1c).

To address the relevance of USP7 in adipogenesis, the expression of this protein was reduced using siRNA-mediated knock-down. Adipogenesis was inhibited by siRNA oligonucleotides against USP7, as illustrated by triglyceride staining with Oil-red-O (Fig. 1d) and FABP4 expression (Fig. 1e). The degree of inhibition correlated with the reduction in USP7 protein expression, with oligonucleotide #12 being the most efficient and oligonucleotide #11 displaying a partial effect. Importantly, USP7 represented not only a major DUB activity in the 3T3-L1 cell culture system (Fig. 1b), but also in mouse white and brown adipose tissue (WAT and BAT, respectively; Fig. 1f). Together these data qualify USP7 as a major and essential deubiquitinating enzyme in mature adipocytes.

USP7 interacts with and deubiquitinates Tip60

Since Tip60 can be polyubiquitinated [24,26] and since USP7 activity is upregulated during adipogenesis (Fig. 1), we investigated whether Tip60 may be a direct USP7 deubiquitination substrate. We therefore first examined the interaction between these two proteins. Cells were co-transfected with HA-tagged Tip60 and myc-tagged USP7, followed by

immunoprecipitation. As shown in Fig. 2a, Tip60 co-immunoprecipitated with myc-tagged USP7 and *vice versa*. As controls,

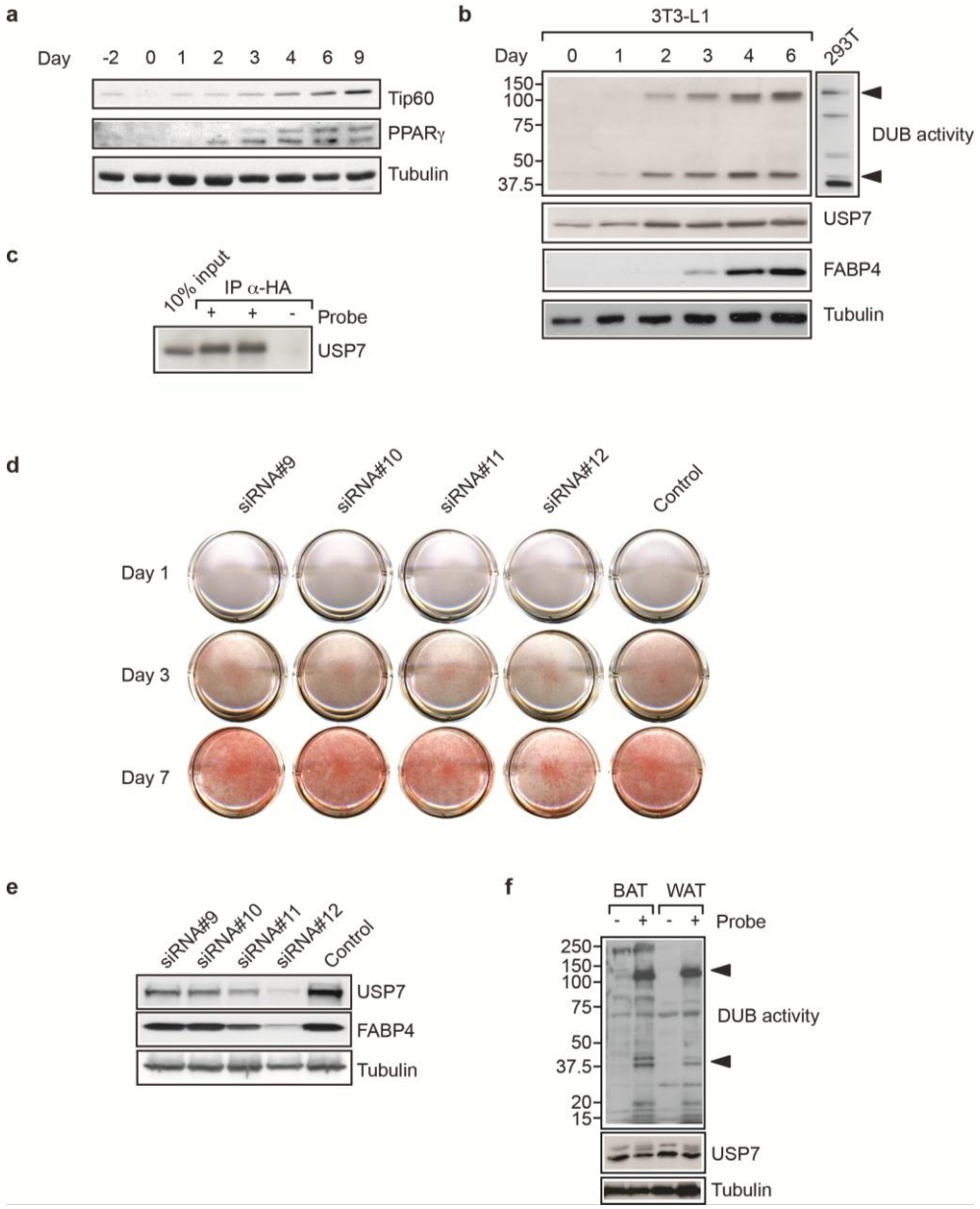

Figure 1. USP7 is essential for adipogenesis. (**a**) Mouse 3T3L1 preadipocytes were differentiated into mature adipocytes and samples were taken at different time points during differentiation. Protein expression levels of Tip60 and PPAR

were determined by Western blot analysis. (**b**) 3T3-L1 cells were differentiated and cells were lysed at different time points. Cells lysates were incubated with a vinyl methyl ester HA-Ub probe to covalently tag active DUBs. DUB activity was detected by Western blotting (anti-HA antibody). Protein expression levels of USP7 were determined by Western blot analysis. As a control for differentiation FABP4 protein levels were analysed. (**c**) Cell lysates of differentiated 3T3-L1 cells (day 6) were incubated with or without HA-Ub probe, DUB activities were immunoprecipitated (anti-HA agarose) and USP7 was detected by Western blotting. Note the difference in mobility between unmodified USP7 (input lane) and USP7 covalently bound to the DUB probe. (**d**) 3T3-L1 cells, transfected with either control or USP7 siRNA oligonucleotides, were subjected to differentiation conditions. At day 0, 3 or 6 of differentiation, cells were fixed and stained for triglycerides using Oil-red-O. Pictures are representative for three independent experiments. (**e**) 3T3-L1 cells were transfected with either control or USP7 siRNA oligonucleotides during differentiation as described under (d). Cell lysates were subjected to Western blot analysis, using antibodies against USP7, FABP4 and tubulin. (**f**) Mouse BAT and WAT were collected, tissues were lysed and DUB activity was determined as described under (b).

immunoprecipitations were performed on cell lysates in which neither or only one of the proteins was present. No co-immunoprecipitations were observed in these cases (Fig. 2a), nor did USP7 co-immunoprecipitate with PPAR (Supplementary Information Fig. S1). More detailed *in vitro* protein-protein interactions studies confirmed the Tip60-USP7 interaction and revealed the importance of the zinc finger and the C-terminal region of Tip60 for the interaction with USP7 (Fig. 2b).

Next we examined the interaction between the endogenous Tip60 and USP7 proteins in 3T3-L1 adipocytes. As both cytoplasmic and nuclear localizations of Tip60 [23,31-33] and USP7 [34-36] have been reported, we first investigated the localization of the proteins in differentiated 3T3-L1 cells. For this an immunofluorescence staining of Tip60 and USP7 was combined with fluorescent staining for intracellular lipid droplets (Nile red) to mark differentiating cells. Tip60 and USP7 both displayed nuclear localization in mature 3T3-L1 adipocytes, while Tip60 was also present in the cytoplasm (Fig. 2c). To further validate the Tip60-USP7 interaction, we performed endogenous co-immunoprecipitation experiments in mature 3T3-L1 cells. Tip60 could be detected after immunoprecipitation with an antibody against USP7, but not with an irrelevant antibody (Gal4) (Fig. 2d). To confirm this direct association between endogenous USP7 and Tip60, an *in situ* proximity ligation

assay (PLA) was performed in 3T3-L1 adipocytes (see Methods). Since a PLA signal can only be obtained when the proteins of interest are in extremely close proximity, this technique enables the detection of direct protein-protein interactions in cells. Association of USP7 and Tip60 was observed and interaction was localized specifically to the nucleus (Fig. 2e).

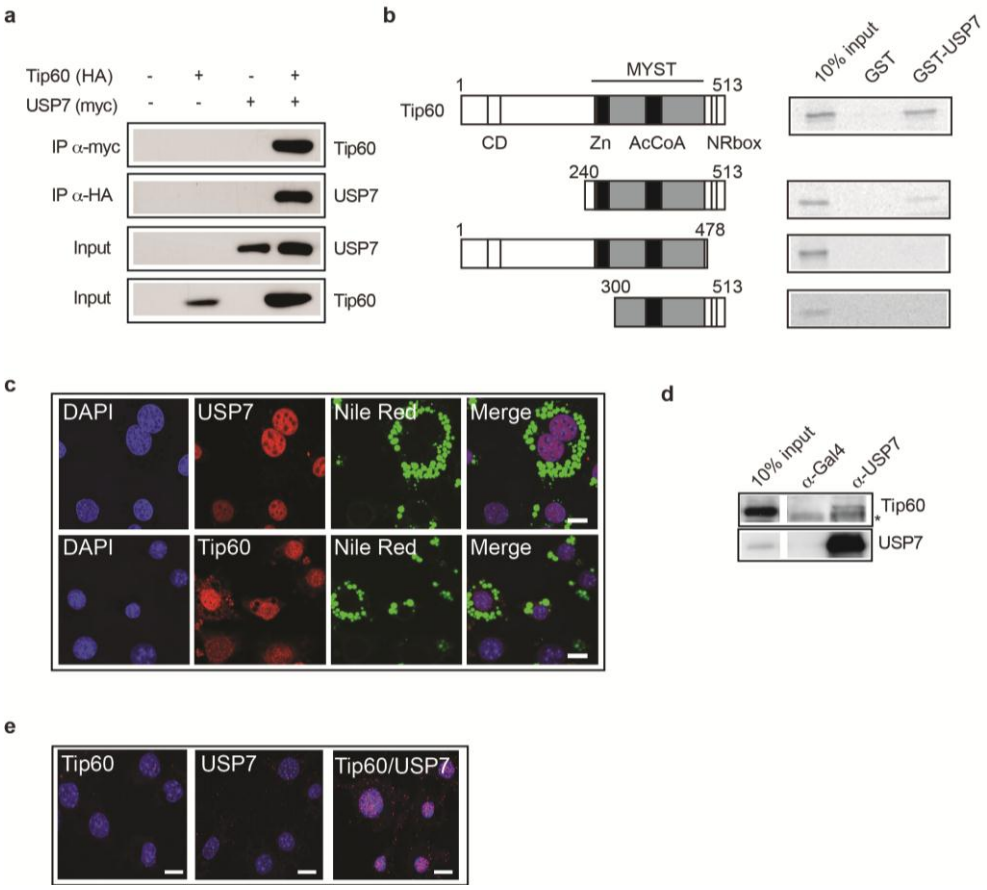

Figure 2. USP7 interacts with Tip60. (**a**) Myc-tagged USP7 and HA-tagged Tip60 were expressed either alone or together in HEK293T cells. After lysis, USP7 and Tip60 were immunoprecipitated with anti-myc and anti-HA antibodies, respectively. Immunoreactive proteins were detected on Western blots by anti-HA and anti-myc antibodies. (**b**) Bacterially expressed and purified GST-USP7 protein was incubated with various [^{35}S]-labelled Tip60 proteins, depicted schematically on the left. Samples were subjected to SDS-PAGE and labelled proteins were visualized by fluorography. (**c**) Representative confocal microscopy images of 3T3-L1 adipocytes. Endogenous Tip60 and USP7 (both red) were visualized

utilizing specific antibodies, differentiated cells were identified with Nile Red (green), Hoechst was used to visualize the nuclei (blue). (**d**) Interaction between endogenous Tip60 and USP7 proteins in differentiated 3T3-L1 cells. USP7 was immunoprecipitated from cell lysates of differentiated 3T3-L1 cells (day 6). Immunoreactive proteins were detected on Western blots by anti-USP7 and anti-Tip60 antibodies. As a control an irrelevant antibody was used (anti-Gal4 antibody). An aspecific band is indicated (*). (**e**) Tip60-USP7 was visualized in 3T3-L1 adipocytes using an *in situ* proximity ligation assay (PLA). Cells were fixed and protein-protein interactions were visualized utilizing anti-Tip60 and anti-USP7 antibodies as described in the Methods section. Punctate staining (red) indicates a Tip60-USP7 interaction as detected by the assay, Hoechst was used to visualize the nuclei (blue).

To further examine the role of USP7 in regulating Tip60 protein expression levels, we tested whether USP7 could directly deubiquitinate Tip60 and thereby increase Tip60 protein levels. We first confirmed that Tip60 can be ubiquitinated by expressing Tip60 together with histidine-tagged ubiquitin in cells, followed by treatment with the proteasome inhibitor MG132. Purification of histidine-tagged proteins followed by Western blotting with an antibody directed against Tip60 revealed high molecular weight species of Tip60, indicative of polyubiquitination (Fig. 3a). Mutation of all seven lysine residues in ubiquitin (7KR), which blocks polybranching of ubiquitin, prevented formation of most of the modified Tip60 forms, confirming that Tip60 is indeed polyubiquitinated (Fig. 3a). To identify which of the 41 lysine residues in Tip60, which are all highly conserved in evolution, were ubiquitinated we isolated ubiquitinated Tip60 from cells, subjected the protein to digestion with several proteases (see Supplementary Information Fig. S2) and identified ubiquitinated sites by mass spectrometry (total coverage 74%; 36 out of 41 lysines covered). We found five high confidence ubiquitin sites and one site with lower confidence (Fig. 3b and Supplementary Information Fig. S2). Substitution of these six lysines by arginines did not significantly alter Tip60 ubiquitination levels (data not shown), suggesting that the protein harbours additional unidentified ubiquitination sites or that Tip60 ubiquitination is promiscuous, as observed in other proteins [37-40]. Mutation of all 41 lysine residues into arginine residues in the Tip60 protein however completely ablated polyubiquitination (Fig. 3c). We therefore conclude that Tip60 can be polyubiquitinated on multiple lysine residues.

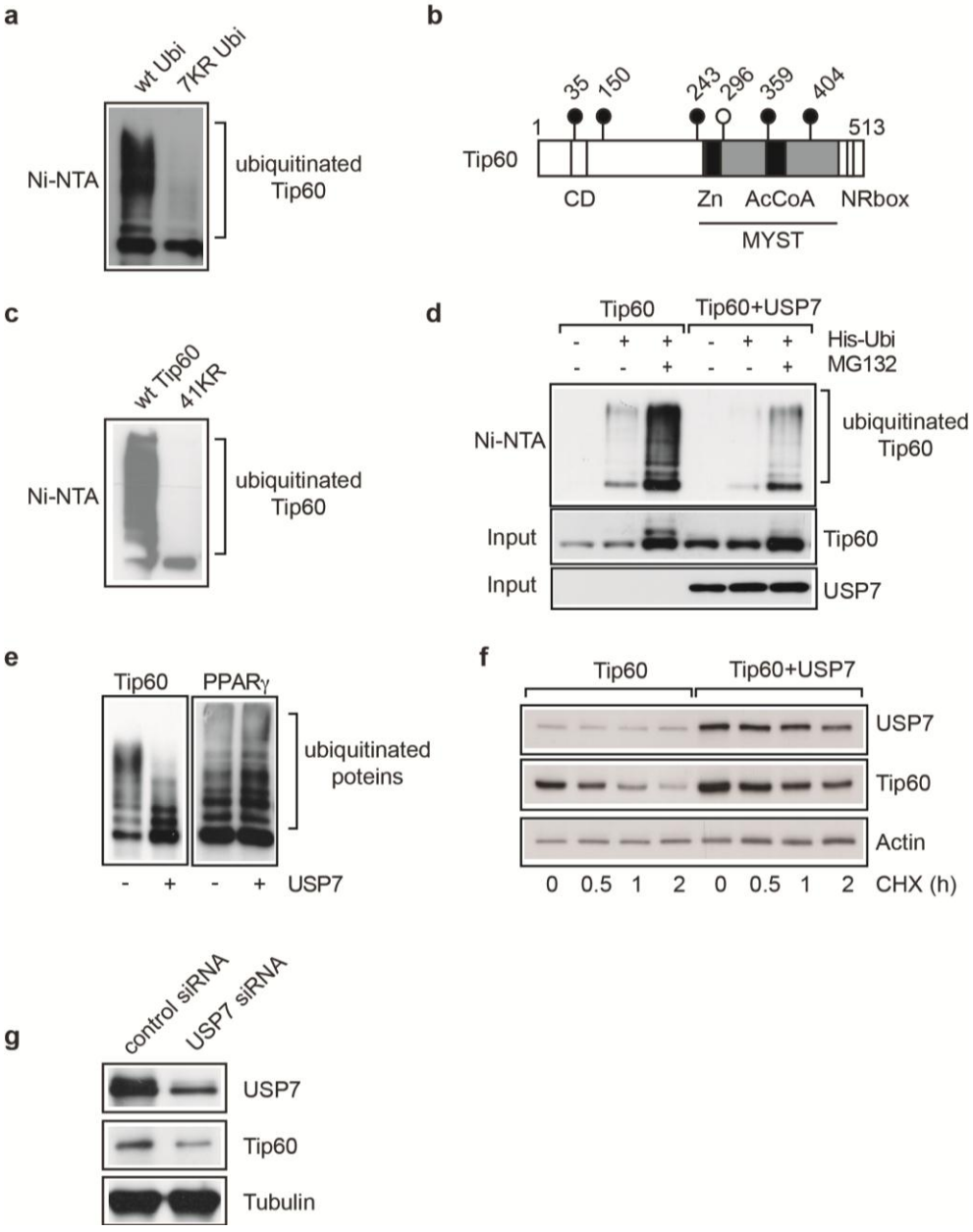

Figure 3. USP7 deubiquitinates Tip60. (**a**) HEK293T cells were transfected with HA-tagged Tip60 expression construct together with wild type or 7KR mutant histidine-tagged ubiquitin (His-ubi) expression construct and treated with MG132 (3 M). Ubiquitinated proteins were isolated by Ni-NTA precipitation and

ubiquitinated Tip60 was detected by Western blotting (anti-HA antibody). (**b**) Schematic representation of the 5 high confidence (filled circles) and one lower confidence (open circle) ubiquitination sites in Tip60 identified by MS/MS (see Supplementary Information Fig. S2). (**c**) HEK293T cells were transfected with wild type HA-tagged Tip60 expression construct or the 41KR mutant together with histidine-tagged ubiquitin (His-ubi) and treated with MG132 (3 M). Ubiquitinated Tip60 was detected as described under (b). (**d**) HEK293T cells were transfected with HA-Tip60 expression vector with or without myc-USP7 expression vector and with or without his-tagged ubiquitin expression vector in the absence or presence of MG132, as indicated. Cells were lysed, ubiquitinated proteins were isolated by Ni-NTA purification and immunoreactive proteins were detected on Western blots by anti-HA, anti-Tip60 and anti-myc antibodies. (**e**) Ubiquitinated HA-Tip60 and HA-PPAR were produced as described under (a), eluted from the Ni-NTA beads with imidazole and incubated with recombinant USP7 enzyme. Tip60 and PPAR were detected by Western blotting (anti-HA antibody). (**f**) HA-Tip60 expression vector was co- transfected with empty vector or USP7 expression vector in 911 cells. Cells were treated with cycloheximide (30 ng/ml), harvested at different time points and subjected to Western blot analysis. (**g**) Mature 3T3-L1 adipocytes were subjected to RNAi-mediated knock down with control or USP7-specific oligonucleotides. Cell lysates were subjected to Western blot analysis, using antibodies against USP7, Tip60 and tubulin.

To investigate whether USP7 can deubiquitinate Tip60, we first performed ubiquitination experiments in the absence or presence of USP7 in living cells. As shown in Figure 3d, inhibition of the proteasome by MG132 resulted in accumulation of ubiquitinated forms of Tip60. Co-expression of USP7 clearly reduced the levels of ubiquitinated Tip60. To exclude the possibility that USP7 affected Tip60 protein stability indirectly, we performed *in vitro* deubiquitination assays. Recombinant USP7 clearly reduced the levels of Tip60 polyubiquitination (Fig. 3e). Importantly, recombinant USP7 displayed substrate specificity *in vitro*, as ubiquitination of PPAR , a well-established ubiquitinated protein [41], was not affected by USP7 activity (Fig. 3e).

Next, we examined the effect of USP7 on Tip60 protein levels. For this, Tip60 was expressed alone or together with USP7 and cells were treated for different periods of time with the protein translation inhibitor cycloheximide. In the absence of USP7, the amount of Tip60 protein gradually decreased in time (Fig. 3f). Co-expression of USP7 significantly increased the steady state level of Tip60, and higher Tip60 protein expression levels were observed at all time points (Fig. 3f). Finally, to examine the role of USP7 in regulating endogenous Tip60 protein levels, USP7 was knocked down in mature 3T3-L1 adipocytes,

using the most effective siRNA oligonucleotide against USP7 (#12; Fig. 1e). USP7 knock down resulted in a marked reduction of Tip60 protein (Fig. 3g), but not mRNA expression (Supplementary Information Fig. S3). Taken together these data show that USP7-mediated deubiquitination regulates Tip60 protein levels.

Tip60 and USP7 regulate common target genes in differentiated 3T3-L1 cells

Having established that USP7 interacts with and deubiquitinates Tip60 (Fig. 2 and 3), it was investigated whether Tip60 and USP7 are functionally linked to adipocyte differentiation. As knock down of USP7 may indirectly reduce Tip60 levels by inhibiting adipogenesis (Fig. 1d and 1e)[25], analysis of the transcriptome under these conditions is likely to reflect differentiation status rather than gene regulation through Tip60 and/or USP7. We therefore performed knock down experiments in differentiated 3T3-L1 adipocytes. Based on previous experiments, the most effective siRNA oligonucleotides against Tip60 (#2; [25] and USP7 (#12; Fig. 1e and 3g) were used. Treatment of mature 3T3-L1 adipocytes with these oligonucleotides resulted in approximately 80% reduced expression of Tip60 and USP7 (Supplementary information, Fig. S3). Microarray analysis revealed that the expression of 1646 and 171 genes were altered after knock down of Tip60 or USP7, respectively (Fig. 4a). A highly significant overlap (p-value $4.46*10^{-40}$) was found between the data sets, consisting of 78 genes that are altered upon Tip60 and USP7 knock down and these genes were either up- (20 genes) or down-regulated (58 genes). Microarray results were validated by quantitative RT-PCR (Supplementary Information Fig. S4). Since USP7 regulates Tip60 protein levels (Fig. 3), knock down of USP7 would be predicted to have the same effect as knock down of Tip60. Indeed, knock down of Tip60 or USP7 altered the expression of 76 of the 78 commonly affected genes in the same direction (Fig. 4b). As >45% of the USP7-regulated genes are also regulated by Tip60 (78 out of 171), our findings suggest that Tip60 is an important mediator of gene regulation by USP7. USP7 seems to play a less dominant role in Tip60-mediated gene regulation, as the proportion of Tip60 target genes that is also regulated by USP7 is much smaller (78 out of 1646). To identify the function(s) of the Tip60-USP7 target genes, we preformed Ingenuity Pathway Analysis with both complete transcriptome data sets. The pathway most clearly affected is the cell cycle, with the majority of

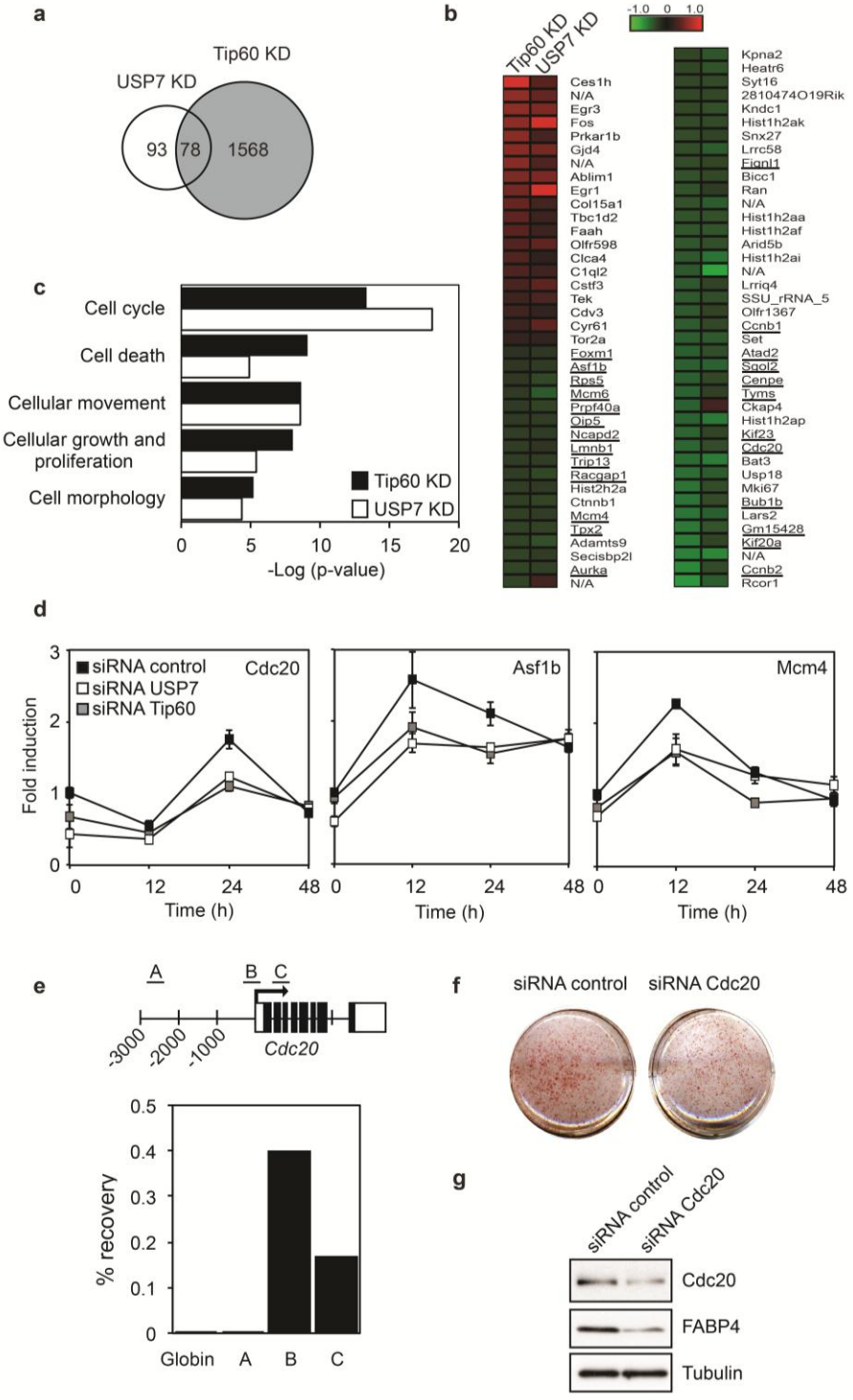
a
Tip60 KD
USP7 KD
93
78
1568
b
Tip60 KD
USP7 KD
-1.0 0 1.0
Ces1h
N/A
Egr3
Fos
Prkar1b
Gjd4
N/A
Ablim1
Egr1
Col15a1
Tbc1d2
Faah
Olfr598
Clca4
C1ql2
Cstf3
Tek
Cdv3
Cyr61
Tor2a
Foxm1
Asf1b
Rps5
Mcm6
Prpf40a
Oip5
Ncapd2
Lmnb1
Trip13
Racgap1
Hist2h2a
Ctnnb1
Mcm4
Tpx2
Adamts9
Secisbp2l
Aurka
N/A
Kpna2
Heatr6
Syt16
2810474O19Rik
Kndc1
Hist1h2ak
Snx27
Lrrc58
Fignl1
Bicc1
Ran
N/A
Hist1h2aa
Hist1h2af
Arid5b
Hist1h2ai
N/A
Lrriq4
SSU_rRNA_5
Olfr1367
Ccnb1
Set
Atad2
Sgol2
Cenpe
Tyms
Ckap4
Hist1h2ap
Kif23
Cdc20
Bat3
Usp18
Mki67
Bub1b
Lars2
Gm15428
Kif20a
N/A
Ccnb2
Rcor1
c
Cell cycle
Cell death
Cellular movement
Cellular growth and proliferation
Cell morphology
Tip60 KD
USP7 KD
0 5 10 15 20
-Log (p-value)
d
siRNA control
siRNA USP7
siRNA Tip60
Cdc20
Asf1b
Mcm4
Fold induction
0 1 2 3
0 12 24 48
Time (h)
e
A
B C
-3000
-2000
-1000
Cdc20
% recovery
0 0.1 0.2 0.3 0.4 0.5
Globin A B C
f
siRNA control
siRNA Cdc20
g
siRNA control
siRNA Cdc20
Cdc20
FABP4
Tubulin

Figure 4. A Tip60-USP7 gene regulation pathway in 3T3-L1 adipogenesis. (**a**) Mature 3T3-L1 adipocytes were subjected to RNAi-mediated knock down with control, USP7- or Tip60-specific oligonucleotides. mRNA expression was assessed by microarray analysis. Shown are the number of genes with a $p<0.05$ after FDR correction and 5000 permutations which were more then 1.14-fold changed after knock down of Tip60 or USP7 compared to control cells. Data were obtained from experiments performed in quadruplicate. (**b**) Heat map showing log2 fold change in gene expression after knockdown of TIP60 or USP7 in differentiated 3T3-L1 cells. Red indicates increased gene expression compared to the control siRNA treatment, whereas green indicates decreased expression. (**c**) Biological functions were identified with the functional analysis of the Ingenuity Pathway Analysis. Only the top 6 significantly regulated molecular and cellular functions are shown. The –log (P-value) shown on the X-axis was calculated by Fischer's exact-test. Functions are included when Fishers exact P-value < 0.05. (**d**) 3T3-L1 cells, transfected with control, Tip60 or USP7 siRNA oligonucleotides, were subjected to differentiation conditions. At different time points, mRNA expression levels of Cdc20, Asf1b and Mcm4 were analysed by quantitative RT-PCR. (**e**) ChIP-PCR for Tip60 was performed in 3T3-L1 cells (day 2 of differentiation) with primers located upstream of the Cdc20 gene (A), in the Cdc20 promoter (B) and in the body of the gene (C), as indicated in the upper panel. As a negative control the arbitrary beta-globin gene was used. Results are indicated as % of immunoprecipitated chromatin compared to the input. (**f**) 3T3-L1 cells, transfected with either control or Cdc20 siRNA oligonucleotides, were subjected to differentiation conditions. At day 6 of differentiation, cells were fixed and stained for triglycerides using Oil-red-O. Pictures are representative for three independent experiments. (**g**) 3T3-L1 cells were transfected with either control or Cdc20 siRNA oligonucleotides during differentiation as described under (e). Cell lysates were subjected to Western blot analysis, using antibodies against Cdc20, FABP4 and tubulin.

genes being down-regulated upon knock down of Tip60 or USP7 (Fig. 4c and Supplementary Information, Fig. S5 and S6). Interestingly, a significant portion of the commonly regulated genes (underlined genes in Fig. 4b), including Cdc20, Asf1b and Mcm4, display similar expression patterns during adipogenesis with a rise in expression around day 1-2, after which expression drops again (Fig. 4d)[42]. Knock down of USP7 or Tip60 blunted the cycling expression pattern of Cdc20, Asf1b and Mcm4, confirming that the expression of these genes is regulated by Tip60 and USP7 (Fig. 4d). Chromatin immunorecipitation analysis revealed that Tip60 binds both at the Cdc20 promoter and within the body of the gene (Fig. 4e), a pattern also observed for other Tip60 target genes in CD4+ T cells [43]. Tip60 could not be detected on an

arbitrary region of the beta-globin gene, which served as a negative control (Fig. 4e).
To address the importance of commonly regulated genes like Cdc20 in adipogenesis, knock down experiments in 3T3-L1 cells were performed. Knock down of Cdc20 resulted in reduced differentiation as assessed by Oil-Red-O staining (Fig. 4f) and FABP4 epxression (Fig. 4g), indicating that Tip60-USP7 target genes play an important role in adipogenesis.

Tip60 and USP7 regulate early adipogenesis

Since 3T3-L1 cells undergo two sequential rounds of mitosis between day 0 and day 2 of differentiation, a process called MCE, and since the common Tip60-USP7 target gene set was enriched for cell cycle genes (Fig. 4b and c), we examined whether Tip60 and USP7 regulate the early steps in adipogenesis. First, an inhibitor that is active against a broad range of DUBs was added at different time points during 3T3-L1 differentiation (Fig. 5a and b; [44,45]). The DUB inhibitor most clearly reduced adipogenesis when added at early time points (day 0-1), while the effects were less pronounced at later stages (Fig. 5c and d). These findings suggest that USP7 (and the low molecular weight DUB; Fig. 1b) predominantly affects early adipogenesis.
Next, the role of Tip60 and USP7 on MCE was addressed more directly by RNAi mediated knock down experiments. In agreement with a previous report [3], 3T3-L1 cells underwent 2 rounds of mitosis, resulting in an almost four-fold increase in cell number at 48h (Fig. 5e). TIP60 and USP7 knock down both impaired MCE, as the number of cells at day 2 was significantly lower than control cells in both cases (Fig. 5e). Terminal differentiation, as assessed by quantification of triglyceride storage at day 6, was also impaired by knock down of Tip60 or USP7 (Fig. 5f), underscoring the importance of MCE for efficient 3T3-L1 adipogenesis. As a control, knock down of PPAR was performed, which did not affect MCE (Fig. 5e), but still inhibited terminal differentiation (Fig. 5f). Taken together, our findings have revealed deubiquitination of a transcriptional coregulator as a key mechanism in the regulation of early adipogenesis.

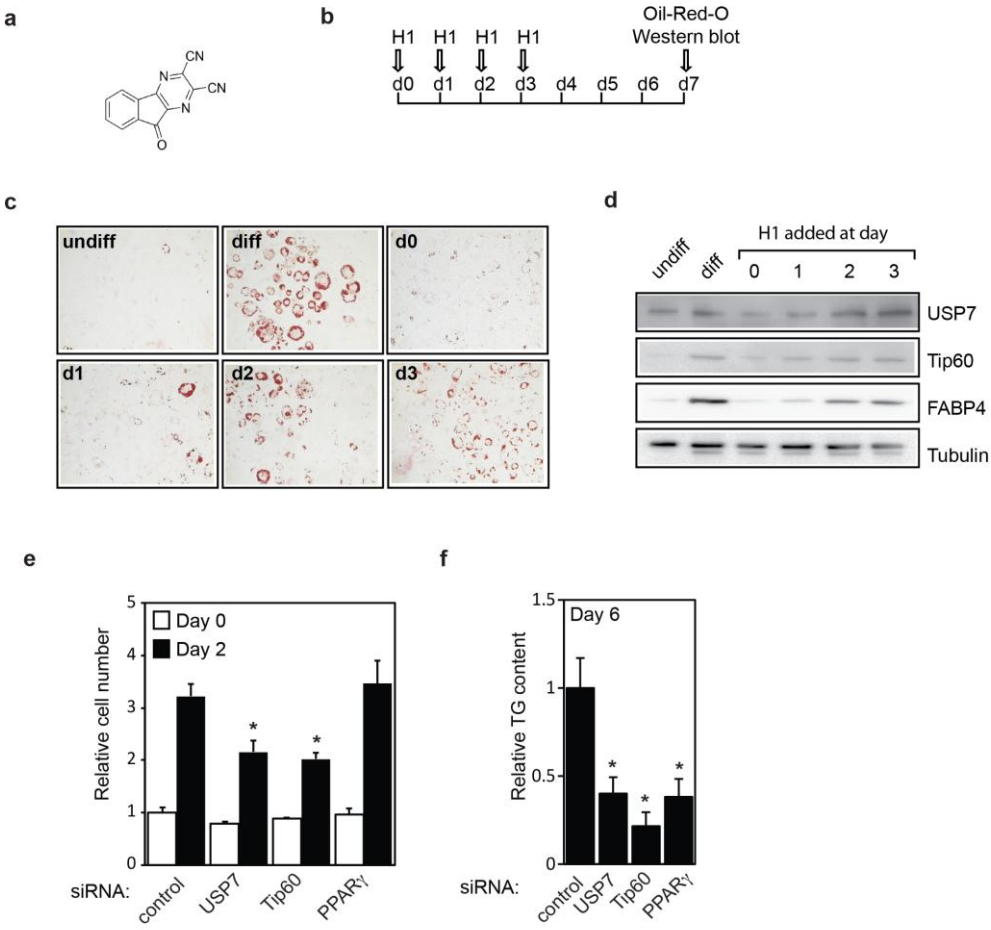

Figure 5. Tip60-USP7 regulate early adipogenesis. (**a**) Structure of the DUB inhibitor. (**b**) Schematic presentation of the DUB inhibitor treatment during 3T3-L1 differentiation. (**c**) 3T3-L1 cells were treated with the DUB inhibitor (2 M) or vehicle during differentiation, as presented in (b). Differentiation was assessed by Oil-red-O staining. (**d**) 3T3-L1 cells were treated with the DUB inhibitor (2 M) or vehicle during differentiation, as presented in (b). Cell lysates were subjected to Western blot analysis, using antibodies against USP7, Tip60, FABP4 and tubulin. (**e**) 3T3-L1 cells were subjected RNAi-mediated knock down of genes indicated and induced to differentiate into adipocytes. Cell numbers were determined at day 0 and at day 2, and the relative cell number was plotted. Asteriks (*) indicate statistically significant differences ($p<0.05$). (**f**) 3T3-L1 cells were subjected RNAi-mediated knock down of genes as described in panel (d), and triglyceride content was determined at day 6 of differentiation. Asteriks (*) indicate statistically significant differences ($p<0.05$).

DISCUSSION

Accumulating evidence indicates that posttranslational modification of transcriptional coregulators presents an important mechanism to regulate fundamental cellular processes. Most studies have focused on modifying enzymes, but demodification may play an equally important role in balancing the transcriptional output. Here we show a direct connection between the transcriptional coregulator Tip60, a histone acetyltransferase, and the deubiquitinase USP7. These two proteins are broadly expressed, fulfil multiple roles in fundamental cellular processes, and are both essential for embryonic development in mice [19,20,46]. Our data show that USP7 is an essential DUB in adipogenesis and reveal the underlying mechanisms to be deubiquitination and stabilization of Tip60.

Our data indicate that enzymatic activity, rather than expression of the USP7 protein, is regulated during adipogenesis. Two molecular mechanisms, that are not mutually exclusive, have been described that can regulate USP7 activity: interaction with specific proteins and PTM. Studies in *D. Melanogaster* and human cells describe the metabolic enzyme GMP synthase (GMPS) to stimulate the ability of USP7 to deubiquitinate histone H2B, and thereby regulate epigenetic silencing [47,48]. Besides GMPS, Sowa *et al.* identified >30 additional proteins USP7 interacting proteins using a global proteomic analysis [35]. In addition, USP7 is subject to ubiquitination [34,49] neddylation [49] and phosphorylation [34], PTM which could all potentially regulate its activity. Future studies are required to establish whether USP7 activity is regulated through protein-protein interactions, PTM or both mechanisms in adipogenesis.

Several lines of evidence suggest that a dominant role of the Tip60-USP7 gene regulation pathway lies in the early stages of adipogenesis. Firstly, the major changes in Tip60 protein expression occur during the first days of differentiation ([25], present study). Secondly, a significant portion of the genes down-regulated after knockdown of either Tip60 or USP7 are mainly involved in mitotic cell cycling. Thirdly, knock down of Tip60 or USP7 inhibited the two rounds of mitosis observed during MCE, an essential early step in 3T3-L1 adipogenesis. Finally, our transcriptome analysis revealed very few “late” adipocyte genes to be critically regulated by Tip60 and/or USP7 (Fig. 4b), despite the fact that Tip60 is recruited to the *Fabp4* and *Plin1* (Perilipin) promoters in mature 3T3-L1 adipocytes [25]. Taken together, these findings suggest

that the critical role of Tip60-USP7 adipogenesis may be the regulation of MCE (day 0-2), while these proteins may be redundant for the maintenance of the adipogenic phenotype. It is tempting to speculate that Tip60 is recruited to its genes by one of the transcription factors that are critical for the early steps in adipogenesis, such as C/EBP [5]. However, our microarray analysis data did not clearly indicate that the Tip60-regulated genes were controlled by a common transcription factor (data not shown). It should be noted that the NuA4/Tip60 complex may also be recruited to target genes through alternative mechanisms, such as H3 lysine 4 trimethylation [50], histone acetylation [51] and RNA polymerase II phosphorylation [43].

Both Tip60 and USP7 have been identified as critical regulators of fundamental cellular processes, such as proliferation and differentiation. Tip60 acetylates p53 and modulates the decision between cell cycle arrest and apoptosis [52-54]. As a deubiquitinase of both Mdm2 and p53, USP7 is involved in the decision-making between cell cycle arrest and proliferation. USP7 and Tip60 share another common target, DNMT1, which maintains DNA methylation patterns after cellular replication. While USP7 stabilizes DNMT1 and facilitates cell cycle progression, Tip60 acetylates DNMT1 and promotes the degradation of DNMT1 by its E3 ligase UHRF1 [55]. Besides playing critical roles in proliferation, both Tip60 and USP7 have recently been implicated in cellular differentiation. Tip60 depletion in *C. elegans* results in premature cell cycle exit and differentiation of one particular cell type, the vulval cells [56]. Secondly, Tip60 was shown to be essential for maintenance of embryonic stem cell (ESC) identity, probably through regulation of the cell cycle and proliferation and by repressing the expression of multiple differentiation genes [50]. Interestingly, Tip60 knock down had only very modest effects on MEF cell cycle distribution and proliferation. USP7 has also been implicated in the regulation of cell differentiation. By deubiquitinating the transcription factor REST, USP7 plays a critical role in the maintenance of neural progenitor cells [57]. While these studies indicate that Tip60 and/or USP7 are important to maintain cells in an undifferentiated state, the differentiation of 3T3-L1 adipocytes and human primary adipocytes critically depends on Tip60 [25,58] and USP7 (current study). Taken together, these studies therefore indicate that Tip60 and USP7 can both inhibit and stimulate proliferation and differentiation, depending on the cellular context. Unravelling the function of Tip60 and USP7 by identifying their

upstream regulators and downstream effectors will therefore not only contribute to our understanding of adipogenesis, but may also help to unravel the fundamental link(s) between cell cycle control and differentiation.

METHODS

Antibodies, plasmids and reagents

The following antibiodies were used: anti-Tip60 (sc-5725 and sc-5727), anti-USP7 (sc-22848), anti-FABP4 (sc-50537) and anti-myc (sc-40), from SantaCruz Biotechnology (SantaCruz, CA), anti-Tip60 (07-038) from Millipore (Billerica, MA), anti-Tip60 (DR1041) from EMD4biosciences (Gibbstown, NJ), anti-USP7 (A300-033A) from Bethyl Laboratories (Montgomery, TX), anti-UCHL3 (3525) from Cell Signalling Technology (Danvers, MA), anti-mouse-HRP (1858413) from Pierce Biotechnologies (Rockford, IL), anti-HA (H9658), anti-actin (A5060), anti-tubulin (T9026) and anti-goat-HRP (A9552) from Sigma-Aldrich (St. Louis, MO). Anti-HA (A2095) or anti-Myc conjugated agarose beads (A7470) were from Sigma-Aldrich (St. Louis, MO). True blot Rabbit IgG beads (00-8800) were from Bioscience (San Diego, CA) and True blot secondary anti-rabbit antibody (18-8816-31) from Pierce Biotechnologies (Rockford, IL). pCDNA-HA-Tip60 [24] and pCL-Myc-USP7 [59] were kind gifts of D. Trouche and M. Maurice, respectively. pGEX2T-USP7 was a kind gift of J. Bartek and J. Lukas [60]. Tip60 deletion constructs were generated by standard cloning procedures [61]. Control siRNA oligonucleotides (D-001810-10) or individual siRNA duplexes against mUSP7 (LQ-052244-01) and mTIP60 (LQ-057795-01) were from Dharmacon (Lafayette, CO). MG-132 (474790) was from EMD4 Biosciences (Gibbstown, NJ). The vinyl methyl ester HA-DUB probe (HAUbVME) and the DUB inhibitor were generated and used as described [28,44,45].

Cell culture, differentiation assays and transfection assays

Human embryonic kidney 293T cell line (HEK293T) and human embryonic retina 911 cell line were maintained in DMEM Glutamax (Invitrogen, Carlsbad, CA) containing 10% foetal calf serum (Invitrogen, Carlsbad, CA), penicillin and streptomycin (Invitrogen, Carlsbad, CA) and transfected as described [25,62]. The 3T3-L1 cell line was cultured and differentiated as described before [25,62]. Differentiation was analysed by Oil-red-O staining and Western blotting as described [25,62], using anti-Tip60 (sc-5725 and DR1041) and anti-USP7 (A300-033A) antibodies. Cells were transfected with siRNA oligonucleotides using RNAiMAX (Invitrogen, Carlsbad, CA) or Amaxa technology (for differentiated 3T3-L1 cells) as described [25,63]. Triglycerides were determined using the Infinity Triglycerides kit (TR 2291-030; Thermo Electron, Louisville, CO)

according to the manufacturers instructions. Results are expressed as TG content relative to control samples and as means ±SD. Differences between means were statistically assessed by a Student's t test in which the limit of significance was set as $p<0.05$.

DUB activity assays and (de)ubiquitination assays

To analyse DUB activity in 3T3-L1 cells, lysates were made in RIPA buffer and cleared by centrifugation. For DUB activity in mouse tissues, epididymal WAT and interscapular BAT were isolated from wild type male C57 Bl/6 mice and lysates were made with M-PER Mammalian Protein Extraction Reagent (Thermo Fischer Scientific, Etten-Leur, The Netherlands) according to the manufacturer's instructions. Lysates were incubated with DUB probe (0.1 g probe/20 g cell lysate) for 1h at 37ºC [27]. Samples were directly subjected to Western blot analysis or DUB activity was first immunoprecipitated using a monoclonal HA-conjugated-agarose antibody.

For *in vivo* (de)ubiquitination assays, cells were transfected with HA-Tip60 and/or myc-USP7 and His-ubi expression plasmids. Twenty-four hours after transfection, cells were incubated o/n with MG132 (3 M), lysed and his-tagged proteins were isolated as described [59,64]. Precipitates were subjected to Western blotting. For *in vitro* deubiquitination assays, his-tagged polyubiquitinated HA-Tip60 was isolated as described above. Recombinant human His6-USP7 (E-519; Boston Biochem, Boston, MA) was added to the substrates for 1 hour at 37ºC in deubiquitination buffer (50 mM Tris PH 7.4, 150 mM NaCl, 10 mM DTT, 5 mM $MgCl_2$). Reactions were stopped by adding 2xSDS sample buffer and analysed by Western blotting.

Mass spectrometry analysis

Proteins were separated by by SDS-PAGE on 4-12% NuPAGE gels (Invitrogen, Carlsbad, CA) and stained with Simply Blue Safestain (Invitrogen, Carlsbad, CA). Bands were excised from gel, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (Roche Applied Science, Almere, The Netherlands) as described [65]. Samples were subjected to nanoflow LC (Eksigent, Dublin, Ireland) using C_{18} reverse phase trap columns (Phenomenex, Utrecht, The Netherlands; column dimensions 2cm x 100 µm, packed in-house) and subsequently separated on C_{18} analytical columns (Reprosil; column dimensions, 20 cm x 50 µm; packed in-house) using a linear gradient

from 0 to 40% B (A = 0.1 M acetic acid; B = 95% (v/v) acetonitrile, 0.1 M acetic acid) in 60 min and at a constant flow rate of 150 nl/min. Column eluate was directly coupled to a LTQ-Orbitrap-XL mass spectrometer (Thermo Fisher Scientific, Breda, The Netherlands) operating in positive mode, using Lock spray internal calibration. Data were processed and subjected to database searches using MASCOT software (Matrix Science, Boston, MA) against Swiss Prot and non-redundant NCBI database, with a 10ppm mass tolerance of precursor and 0.8Da for the fragment ion.

Immunoprecipitation assays

Immunoprecipitation experiments were performed as described [25] using anti-HA- or anti-Myc-conjugated agarose. Precipitates were subjected to Western blotting using antibodies against myc- and HA-tags respectively. Endogenous co-immunoprecipitation experiments on differentiated 3T3-L1 cells were performed similarly, using anti-Tip60 (DR1041) antibody, True blot Rabbit IgG beads and True blot secondary anti-rabbit antibody (Pierce Biotechnologies, Rockford, IL).

Immunofluorescence microscopy and proximity ligation assay (PLA)

For immunofluorescence staining, 3T3-L1 cells were plated on glass coverslips and differentiated for 5 days. Subsequently, cells were fixed with 4% paraformaldehyde (20', RT) and permeabilized in PBS supplemented with 0.5% Triton (10'). After 30' incubation in blocking buffer, cells were stained with primary antibodies (anti-Tip60 (DR1041) and anti-USP7 (sc-22848)) for 2h at room temperature, then incubated with secondary fluorochrome-conjugated antibodies. After several washes, coverslips were incubated Nile-Red and Hoechst, washed with PBS, mounted in Mowiol and analyzed with an LSM710 Met confocal microscope (Carl Zeiss, Jena, Germany).

PLA detection was performed using the Duolink II kit (Olink bioscience, Uppsala, Sweden) according to the manufacturer's protocol. In short, differentiated 3T3-L1 cells were enabled to adhere to coverslips and subsequently washed with PBS and fixed with 4% formaldehyde for 20 minutes. Afterwards, the samples were permeabilized with 0.2% triton X-100 for 5 minutes and then incubated for 30 minutes with blocking buffer (10% normal human serum in PBS). After blocking, cells were incubated O/N at 4°C with anti-Tip60 antibody (07-038) and anti-USP7

antibody (sc-22848) in blocking buffer containing 10% normal human serum. Cells were washed three times with PBS, followed by PLA according to manufacturer's protocol. Coverslips were mounted and analysed as described above.

GST pull down assays

Recombinant Tip60 constructs in the pCDNA3 expression vector were transcribed and translated *in vitro* in reticulolysate in the presence of [^{35}S]-methionine according to manufacturers protocol (TNT T7 Quick Coupled Transcription /Translation Kit; Promega, Leiden, The Netherlands). Rosetta pLysS competent bacteria (EMD4 Biosciences, Gibbstown, NJ) were transformed with GST-USP7 expression plasmid [60]. GST fusion proteins were purified as described earlier [66]. GST-USP7 protein were incubated with [^{35}S]-labelled proteins in NETN buffer (20mM Tris pH 8.0, 400mM NaCl, 1mM EDTA, 0.5% NP40) containing protease inhibitors (Complete; Roche Applied Science, Almere, The Netherlands). Samples were subsequently washed and subjected to SDS-PAGE. Gels were enhanced with Amplify (Amersham), dried and visualized by fluorography.

Chromatin immunprecipitation (ChIP)-PCR analysis

For chromatin preparation, 10^8 3T3-L1 (2 days of differentiation) were crosslinked with 2 mM disuccinimidyl glutarate (Thermo Fisher Scientific, Etten-Leur, The Netherlands) and 1% formaldehyde as previously described [67], nuclei were isolated in lysis buffer (10 mM Tris-Cl pH 7.5, 10 mM NaCl, 3 mM MgCl2, 0.5% Igepal) and lysed in pre-immunoprecipitation buffer (10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$ and 1 mM $CaCl_2$). Chromatin was sheared using a Bioruptor (Diagenode, Denville, USA) and chromatin was precipitated with 5 µg anti-Tip60 antibody (sc-5727) and protG-sepharose beads (Sigma-Aldrich, St. Louis, USA). De-crosslinked and precipitated chromatin was dissolved in 100 µl water and for PCR and 5 µl of DNA solution was used for qPCR analysis. The primers used for qPCR were:

5'-ACAGAGAACCACCTGCCACT-3' (Cdc20_A_fw),
5'-AATGTGTCTGCCTTCGGAGT-3' (Cdc20_A_rev),
5'-GGGATTTCCTTCCAAATGCT-3' (Cdc20_B_fw),
5'-TCATTGGCTCCTTCAAAACC-3' (Cdc20_B_rev),
5'-GAGGCTGGTGTCAGTCCTTC-3' (Cdc20_C_fw),
5'-GGTAGCCTGTGGCAAGAGAG-3' (Cdc20_C_rev),

5'-CCTGCCCTCTCTATCCTGTC-3' (βglobin_fw) and
5'-GCAAATGTGTTGCCAAAAAG-3' (βglobin_rev).

RNA isolation, microarray analysis and qRT-PCR

Differentiated 3T3-L1 cells were transfected with Amaxa technology as described [63]. Two days after transfection cells were washed twice with PBS twice and lysed in 0.5ml Trizol (Invitrogen). Samples were incubated at room temperature for 5', 0.1ml chloroform was added, vortexed and centrifuged for 10 minutes at 8000 rpm at 4°C. The pellet was washed with 70% ethanol and dissolved in water. DNAse treatment and purification was performed with RNeasy kit (Qiagen, Venlo, The Netherlands).

Microarrays used were Mouse Whole Genome Gene Expression Microarrays V1 (Agilent Technologies, Belgium) representing 41174 *M. musculus* 60-mer probes in a 4x44K layout. RNA amplifications and labelling were performed as described in Roepman *et al.* [68] on an automated system (Caliper Life Sciences NV/SA, Belgium) with 3 g total RNA from each sample. Hybridizations were done on an automated hybridization station (HS4800PRO system with QuadChambers; Tecan Benelux B.V.B.A.) using 1000 ng labelled cRNA per channel according to van de Peppel *et al.* [69]. Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery), printtip Loess normalization was performed [70] on mean spot-intensities. Data was analysed using MAANOVA [71]. In a fixed effect analysis, sample, array and dye effects were modelled. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Genes (based on unique ENSMUSG gene IDs) with $p < 0.05$ after determination of false discovery rate (FDR) were considered significantly changed. A cut-off fold change > ±1.14 was used. Hypergeometric testing was performed to identify significance of overlap. Microarray data were analysed with Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenutiy.com). Functional analysis identified biological functions that were differentially expressed between knockout and wild type cells. Genes with $p < 0.05$ after FDR that met the cut-off fold change > ±1.14, and that were associated with a biological function in the Ingenuity Pathway Knowledge Base were included for the analysis. Fisher's exact-test was used to calculate a P-value for each biological function, and functions

were considered to be differentially expressed between genotypes when the Fishers exact P-value < 0.05. In addition, biological interaction networks among Tip60 and USP7 regulated genes were identified. A statistical likelihood approach was used to identify the significance of the generated networks. Microarray data have been submitted to ArrayExpress and GEO, accession numbers are pending.

For qRT-PCR analysis, three independent samples of total RNA were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Gene expression levels were determined by quantitative real-time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to *TFIIb* expression. The primers used were TFIIb forward primer 5'-TCCTCCTCAGACCGCTTTT-3' and reverse primer 5'-CCTGGTTCATCATCGCTAATC-3'; Cdc20 forward primer 5'-GCTCAAAGGACACACAGCAC-3' and reverse primer 5'-GCCACAACCGTAGAGTCTCA-3'; Asf1b forward primer 5'-CTGACGACCTGGAGTGGAAG-3' and reverse primer 5'-AGGTGCAGGTGATGAGAACC-3'; Mcm4 forward primer 5'-GTCACACTGGGAGGGGTAAA-3' and reverse primer 5'-CACTGGAGCCTTTTCCAGAC-3'; Egr1 forward primer 5'-CTGACCACAGAGTCCTTTTCTG-3' and reverse primer 5'-GTTCAGGCCACAAAGTGTTG-3'; Fos forward primer 5'-CCAAGCGGAGACAGATCAACT-3' and reverse primer 5'-TGGGCTGCCAAAATAAACTC-3'; Aurka forward primer 5'-CATGCTCCATCTTCCAGGAG-3' and reverse primer 5'-AGGCATCCCCACTAGGAACT-3'. Results are expressed as mRNA expression relative to control samples and as means ±SD. Differences between means were statistically assessed by a Student's t test in which the limit of significance was set as $p<0.05$.

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AUTHOR CONTRIBUTION STATEMENT
Y.G., A.K., O.B., I.T., S.F.G., J.L., N.H., D.L., C.R.B. and A.B.B. designed and performed experiments and analysed the data; R.B., F.C.P., P.J.C., A.B.B., H.O. and E.K. supervised the study; Y.G. and E.K. wrote the manuscript.

REFERENCES

1 Green, H. & Kehinde, O. Sublines of mouse 3T3 cells that accumulate lipid. *Cell* **1**, 113-116 (1974).

2 Poulos, S. P., Dodson, M. V. & Hausman, G. J. Cell line models for differentiation: preadipocytes and adipocytes. *Exp.Biol.Med.(Maywood.)* **235**, 1185-1193 (2010).

3 Tang, Q. Q., Otto, T. C. & Lane, M. D. Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc.Natl.Acad.Sci.U.S.A* **100**, 44-49 (2003).

4 Farmer, S. R. Transcriptional control of adipocyte formation. *Cell Metab* **4**, 263-273 (2006).

5 Rosen, E. D. & MacDougald, O. A. Adipocyte differentiation from the inside out. *Nat.Rev.Mol.Cell Biol.* **7**, 885-896 (2006).

6 van Beekum, O., Fleskens, V. & Kalkhoven, E. Posttranslational modifications of PPAR-gamma: fine-tuning the metabolic master regulator. *Obesity.(Silver.Spring).* **17**, 213-219 (2009).

7 Nerlov, C. C/EBPs: recipients of extracellular signals through proteome modulation. *Curr.Opin.Cell Biol.* **20**, 180-185 (2008).

8 Han, S. J., Lonard, D. M. & O'Malley, B. W. Multi-modulation of nuclear receptor coactivators through posttranslational modifications. *Trends Endocrinol.Metab* **20**, 8-15 (2009).

9 Louet, J. F. *et al.* Oncogenic steroid receptor coactivator-3 is a key regulator of the white adipogenic program. *Proc.Natl.Acad.Sci.U.S.A* **103**, 17868-17873 (2006).

10 Louet, J. F. & O'Malley, B. W. Coregulators in adipogenesis: what could we learn from the SRC (p160) coactivator family? *Cell Cycle* **6**, 2448-2452 (2007).

11 Hartig, S. M., He, B., Long, W., Buehrer, B. M. & Mancini, M. A. Homeostatic levels of SRC-2 and SRC-3 promote early human adipogenesis. *J.Cell Biol.* **192**, 55-67 (2011).

12 York, B. & O'Malley, B. W. Steroid receptor coactivator (SRC) family: masters of systems biology. *J.Biol.Chem.* **285**, 38743-38750 (2010).

13 Utley, R. T. & Cote, J. The MYST family of histone acetyltransferases. *Curr.Top.Microbiol.Immunol.* **274**, 203-236 (2003).

14 Yang, X. J. The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. *Nucleic Acids Res.* **32**, 959-976 (2004).

15 Doyon, Y. *et al.* ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Mol.Cell* **21**, 51-64 (2006).
16 Doyon, Y., Selleck, W., Lane, W. S., Tan, S. & Cote, J. Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. *Mol.Cell Biol.* **24**, 1884-1896 (2004).
17 Sapountzi, V., Logan, I. R. & Robson, C. N. Cellular functions of TIP60. *Int.J.Biochem.Cell Biol.* **38**, 1496-1509 (2006).
18 Squatrito, M., Gorrini, C. & Amati, B. Tip60 in DNA damage response and growth control: many tricks in one HAT. *Trends Cell Biol.* **16**, 433-442 (2006).
19 Gorrini, C. *et al.* Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. *Nature* **448**, 1063-1067 (2007).
20 Hu, Y. *et al.* Homozygous disruption of the Tip60 gene causes early embryonic lethality. *Dev.Dyn.* **238**, 2912-2921 (2009).
21 Zhu, X., Singh, N., Donnelly, C., Boimel, P. & Elefant, F. The cloning and characterization of the histone acetyltransferase human homolog Dmel\TIP60 in Drosophila melanogaster: Dmel\TIP60 is essential for multicellular development. *Genetics* **175**, 1229-1240 (2007).
22 Lemercier, C. *et al.* Tip60 acetyltransferase activity is controlled by phosphorylation. *J.Biol.Chem.* **278**, 4713-4718 (2003).
23 Cheng, Z. *et al.* Functional characterization of TIP60 sumoylation in UV-irradiated DNA damage response. *Oncogene.* **27**, 931-941 (2008).
24 Legube, G. *et al.* Tip60 is targeted to proteasome-mediated degradation by Mdm2 and accumulates after UV irradiation. *EMBO J.* **21**, 1704-1712 (2002).
25 van Beekum, O. *et al.* The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARgamma. *Endocrinology* **149**, 1840-1849 (2008).
26 Col, E. *et al.* HIV-1 Tat targets Tip60 to impair the apoptotic cell response to genotoxic stresses. *EMBO J.* **24**, 2634-2645 (2005).
27 Ovaa, H. *et al.* Activity-based ubiquitin-specific protease (USP) profiling of virus-infected and malignant human cells. *Proc.Natl.Acad.Sci.U.S.A* **101**, 2253-2258 (2004).

28 Borodovsky, A. *et al.* Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem.Biol.* **9**, 1149-1159 (2002).
29 Brooks, C. L. & Gu, W. Dynamics in the p53-Mdm2 ubiquitination pathway. *Cell Cycle* **3**, 895-899 (2004).
30 Wade, M., Wang, Y. V. & Wahl, G. M. The p53 orchestra: Mdm2 and Mdmx set the tone. *Trends Cell Biol.* **20**, 299-309 (2010).
31 Ran, Q. & Pereira-Smith, O. M. Identification of an alternatively spliced form of the Tat interactive protein (Tip60), Tip60(beta). *Gene* **258**, 141-146 (2000).
32 Halkidou, K. *et al.* Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. *Oncogene* **22**, 2466-2477 (2003).
33 McAllister, D., Merlo, X. & Lough, J. Characterization and expression of the mouse tat interactive protein 60 kD (TIP60) gene. *Gene.* **289**, 169-176 (2002).
34 Fernandez-Montalvan, A. *et al.* Biochemical characterization of USP7 reveals post-translational modification sites and structural requirements for substrate processing and subcellular localization. *FEBS J.* **274**, 4256-4270 (2007).
35 Sowa, M. E., Bennett, E. J., Gygi, S. P. & Harper, J. W. Defining the human deubiquitinating enzyme interaction landscape. *Cell.* **138**, 389-403 (2009).
36 Maertens, G. N., El Messaoudi-Aubert, S., Elderkin, S., Hiom, K. & Peters, G. Ubiquitin-specific proteases 7 and 11 modulate Polycomb regulation of the INK4a tumour suppressor. *EMBO J.* **29**, 2553-2565 (2010).
37 Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S. & Sorkin, A. Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Mol.Cell* **21**, 737-748 (2006).
38 Lee, J. S., Hong, U. S., Lee, T. H., Yoon, S. K. & Yoon, J. B. Mass spectrometric analysis of tumor necrosis factor receptor-associated factor 1 ubiquitination mediated by cellular inhibitor of apoptosis 2. *Proteomics.* **4**, 3376-3382 (2004).
39 Lu, Y. *et al.* Ubiquitination and proteasome-mediated degradation of BRCA1 and BARD1 during steroidogenesis in human ovarian granulosa cells. *Mol.Endocrinol.* **21**, 651-663 (2007).

40 Meray, R. K. & Lansbury, P. T., Jr. Reversible monoubiquitination regulates the Parkinson disease-associated ubiquitin hydrolase UCH-L1. *J.Biol.Chem.* **282**, 10567-10575 (2007).
41 Hauser, S. *et al.* Degradation of the peroxisome proliferator-activated receptor gamma is linked to ligand-dependent activation. *J.Biol.Chem.* **275**, 18527-18533 (2000).
42 Mikkelsen, T. S. *et al.* Comparative epigenomic analysis of murine and human adipogenesis. *Cell* **143**, 156-169 (2010).
43 Wang, Z. *et al.* Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell* **138**, 1019-1031 (2009).
44 Colombo, M. *et al.* Synthesis and biological evaluation of 9-oxo-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile analogues as potential inhibitors of deubiquitinating enzymes. *ChemMedChem.* **5**, 552-558 (2010).
45 Guedat, P. *et al.* Patent WO 2007/017758 A2. (2007).
46 Kon, N. *et al.* Inactivation of HAUSP in vivo modulates p53 function. *Oncogene.* **29**, 1270-1279 (2010).
47 van der Knaap, J. A. *et al.* GMP synthetase stimulates histone H2B deubiquitylation by the epigenetic silencer USP7. *Mol.Cell.* **17**, 695-707 (2005).
48 Sarkari, F. *et al.* EBNA1-mediated recruitment of a histone H2B deubiquitylating complex to the Epstein-Barr virus latent origin of DNA replication. *PLoS Pathog* **5**, e1000624, doi:10.1371/journal.ppat.1000624 (2009).
49 Lee, H. J., Kim, M. S., Kim, Y. K., Oh, Y. K. & Baek, K. H. HAUSP, a deubiquitinating enzyme for p53, is polyubiquitinated, polyneddylated, and dimerized. *FEBS Lett.* **579**, 4867-4872 (2005).
50 Fazzio, T. G., Huff, J. T. & Panning, B. An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. *Cell* **134**, 162-174 (2008).
51 Shi, X. *et al.* ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* **442**, 96-99 (2006).
52 Sykes, S. M. *et al.* Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol.Cell* **24**, 841-851 (2006).
53 Tang, Y., Luo, J., Zhang, W. & Gu, W. Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol.Cell* **24**, 827-839 (2006).

54 Li, M. *et al.* Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416**, 648-653 (2002).
55 Du, Z. *et al.* DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. *Sci.Signal.* **3**, ra80 (2010).
56 Ceol, C. J. & Horvitz, H. R. A new class of C. elegans synMuv genes implicates a Tip60/NuA4-like HAT complex as a negative regulator of Ras signaling. *Dev.Cell* **6**, 563-576 (2004).
57 Huang, Z. *et al.* Deubiquitylase HAUSP stabilizes REST and promotes maintenance of neural progenitor cells. *Nat.Cell Biol.* **13**, 142-152 (2011).
58 Gronniger, E. *et al.* A new protocol for functional analysis of adipogenesis using reverse transfection technology and time-lapse video microscopy. *Cell Biol.Int.* **34**, 737-746 (2010).
59 Meulmeester, E. *et al.* Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Mol.Cell.* **18**, 565-576 (2005).
60 Faustrup, H., Bekker-Jensen, S., Bartek, J., Lukas, J. & Mailand, N. USP7 counteracts SCFbetaTrCP- but not APCCdh1-mediated proteolysis of Claspin. *J.Cell Biol.* **184**, 13-19 (2009).
61 Ausubel, F. M. *et al. Current Protocols in Molecular Biology*. (John Wiley & Sons, 1993).
62 Jeninga, E. H. *et al.* Impaired peroxisome proliferator-activated receptor gamma function through mutation of a conserved salt bridge (R425C) in familial partial lipodystrophy. *Mol.Endocrinol.* **21**, 1049-1065 (2007).
63 Jeninga, E. H. *et al.* Peroxisome proliferator-activated receptor gamma regulates expression of the anti-lipolytic G-protein-coupled receptor 81 (GPR81/Gpr81). *J.Biol.Chem.* **284**, 26385-26393 (2009).
64 Stad, R. *et al.* Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. *EMBO Rep.* **2**, 1029-1034 (2001).
65 Wilm, M. *et al.* Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**, 466-469 (1996).
66 Kalkhoven, E., Valentine, J. E., Heery, D. M. & Parker, M. G. Isoforms of steroid receptor co-activator 1 differ in their ability to potentiate transcription by the oestrogen receptor. *EMBO J.* **17**, 232-243 (1998).

67 Nowak, D. E., Tian, B. & Brasier, A. R. Two-step cross-linking method for identification of NF-kappaB gene network by chromatin immunoprecipitation. *Biotechniques* **39**, 715-725 (2005).

68 Roepman, P. *et al.* An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat.Genet.* **37**, 182-186 (2005).

69 van de Peppel, J. *et al.* Monitoring global messenger RNA changes in externally controlled microarray experiments. *EMBO Rep.* **4**, 387-393 (2003).

70 Yang, Y. H. *et al.* Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res.* **30**, e15 (2002).

71 Wu, Y. H., Kerr, M. K. & Churchill, G. A. in *The analysis of gene expression data: methods and software* (2002).

SUPPLEMENTARY INFORMATION

EARLY ADIPOGENESIS IS REGULATED THROUGH USP7-MEDIATED DEUBIQUITINATION OF THE HISTONE ACETYLTRANSFERASE TIP60

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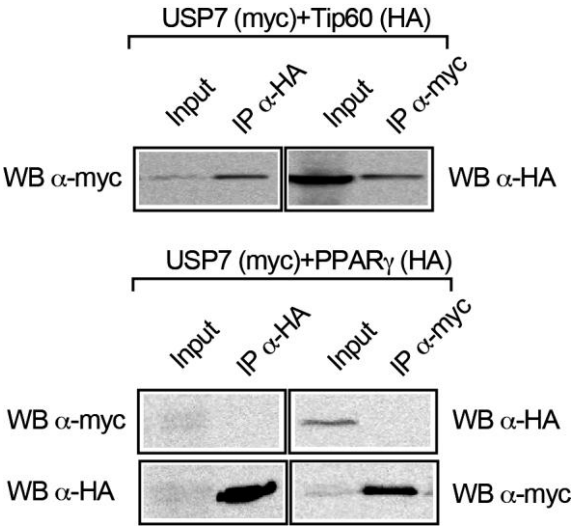
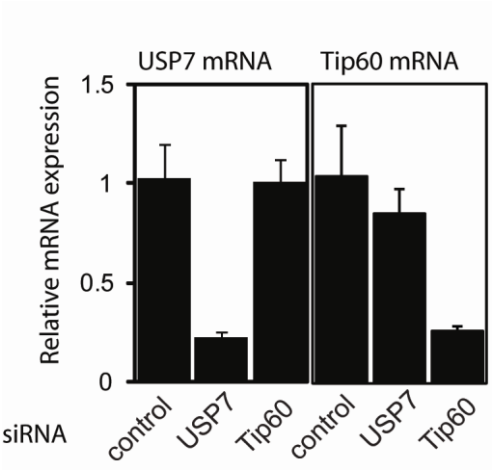


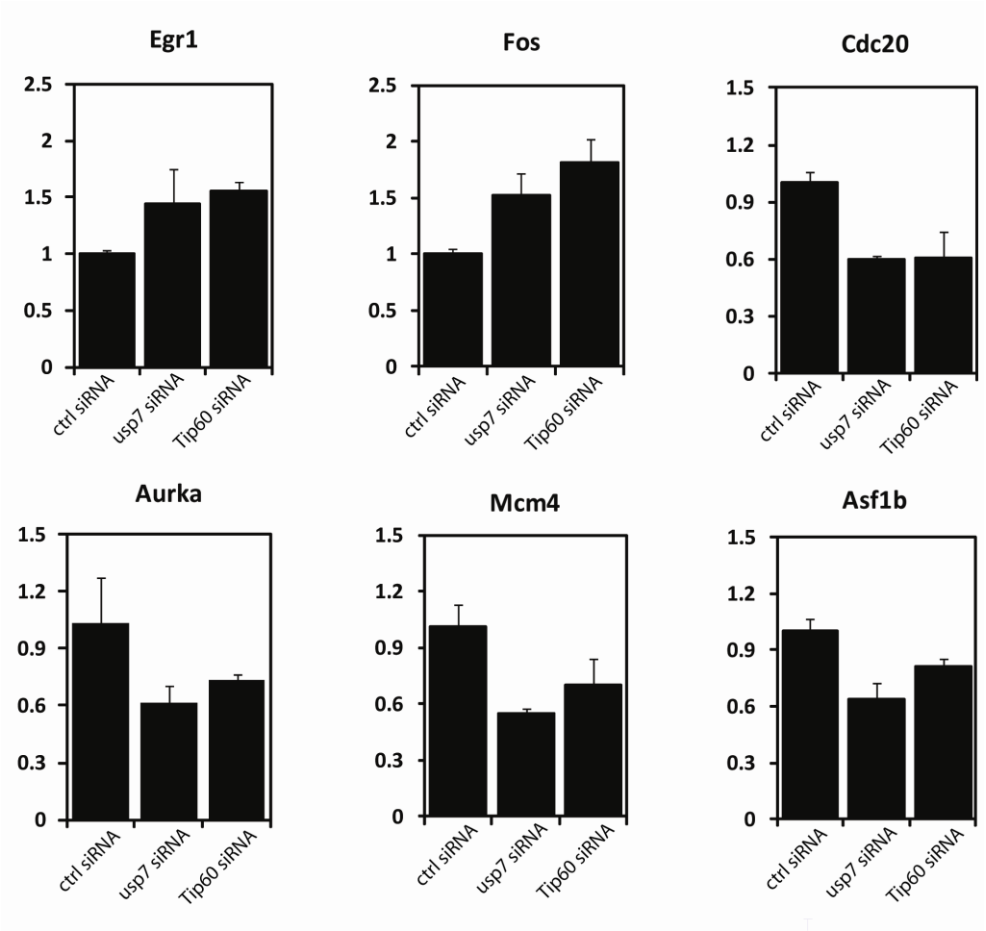
Supplementary Figure S1 Co-immunoprecipitation experiments
Myc-tagged USP7 and HA-tagged Tip60 or HA-tagged PPAR were expressed in HEK293T cells. After lysis, USP7 and Tip60 or PPAR were immunoprecipitated with anti-myc and anti-HA antibodies, respectively. Immunoreactive proteins were detected on Western blots by anti-HA and anti-myc antibodies.

aa position	PTM	Peptide sequence	Peptide AA position	Ionscore	Enzymes
35	GlyGly	ILSV**K**DISGR	31 - 40	65	Trypsin + V8
150	GlyGly	**K**VEVVSPATPVPSETAPA	150 - 167	62	Trypsin + elastase
243	GlyGly	L**K**PWYFSPYPQE	242 - 253	52	Trypsin + V8
296	GlyGly	**K**GTISFFE	296 - 303	19	Trypsin + V8
359	GlyGly	E**K**ESTEDYNVA	358 - 368	30	Trypsin + elastase
404	GlyGly	TGTPE**K**PLSDLGLL	399 - 412	41	Trypsin + elastase
404	GlyGly	TGTPE**K**PLSDLGLLSYR	399 - 415	31	Trypsin + V8
404	GlyGly	TGTPE**K**PLSDLGLL	399 - 412	56	Trypsin + elastase
404	GlyGly	TGTPE**K**PLSDLGLLSYR	399 - 415	37	Trypsin + V8
404	GlyGly	TGTPE**K**PLSD	399 - 408	43	Trypsin + V8

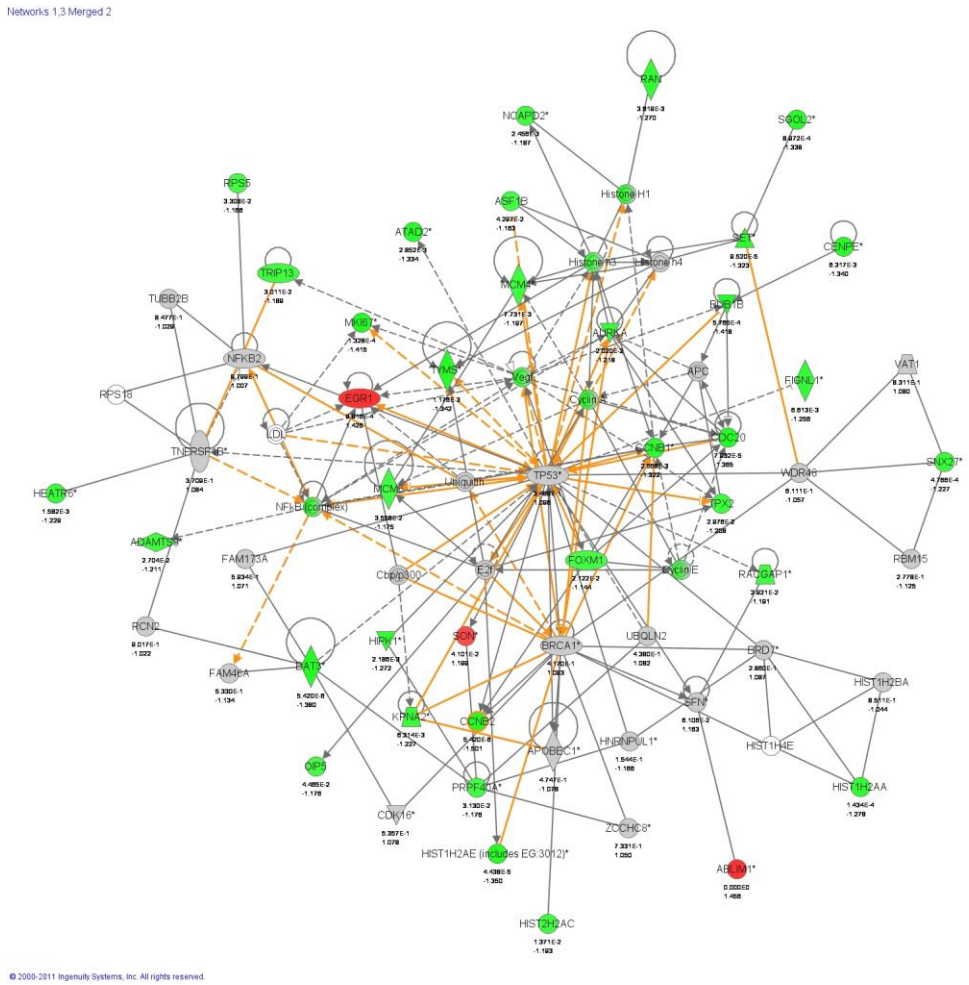
Supplementary Figure S2 Identification of ubiquitin sites in Tip60. Indicated are the amino acid (aa) positions, the posttranslational modifications (PTM), the peptide sequence, the position of the peptide in the Tip60 protein sequence (NP_006379.2), the ionscore and the enzymes used for the protein digestion.



Supplementary Figure S3 mRNA expression of Tip60 and USP7 after RNAi-mediated knock down.
Mature 3T3-L1 adipocytes were subjected to RNAi-mediated knock down with control, USP7- or Tip60-specific oligonucleotides. mRNA expression of Tip60 and USP7 was assessed by qRT-PCR.

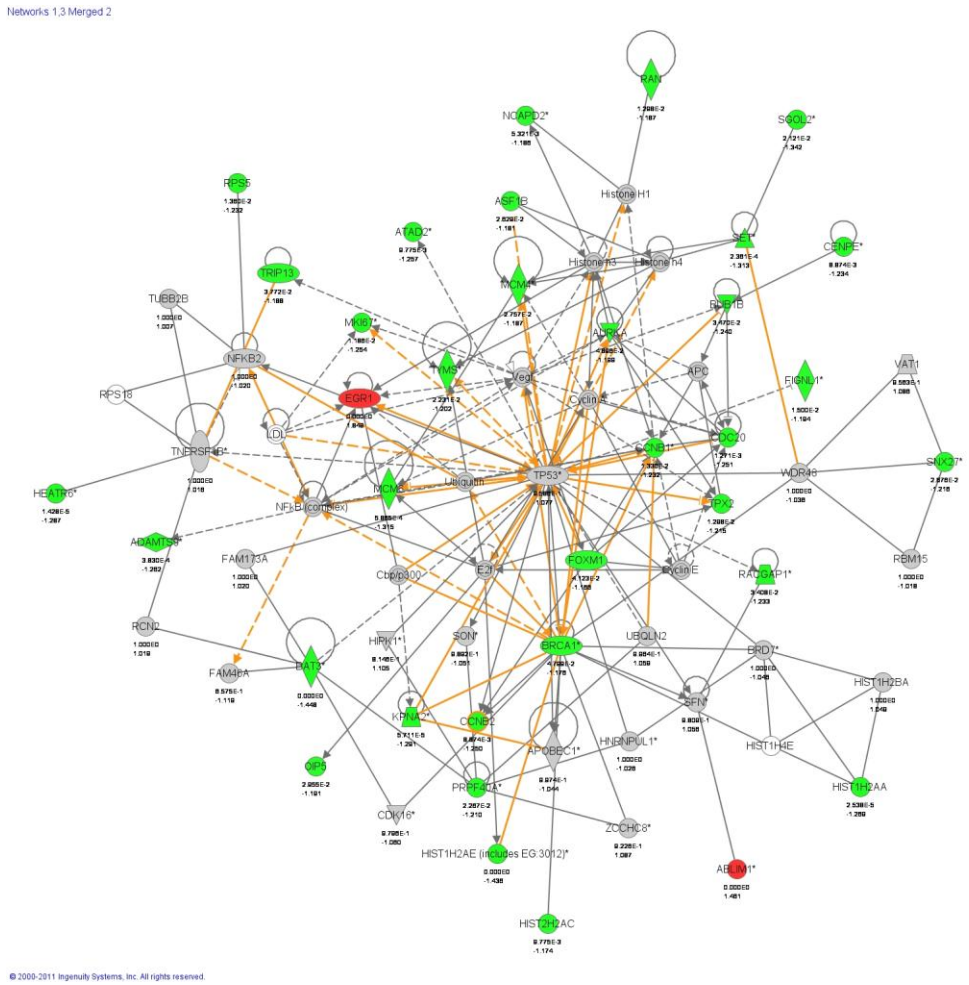


Supplementary figure S4 mRNA expression of selected genes after RNAi-mediated knock down.
RNA was isolated and relative mRNA levels of Egr1, Fos, Cdc20, Mcm4, and Asf1b were analyzed using quantitative RT-PCR. Data are represented as mean±SEM values normalized to a house keeping gene. At least 3 experiments were performed with technical duplicates. All Tip60 and USP7 knock down treatments resulted in significant changes in mRNA expression ($P<0.05$).



Supplementary figure S5 Network analysis of 3T3-L1 transcriptomes after Tip60 knock down.

Ingenuity network explorer was used to detect for top scored biological interaction networks after Tip60 knock down. The intensity of the colors indicates the degree of up or down-regulation, respectively; a greater intensity represents a higher degree of regulation. Red represents up-regulation and green represents down-regulation.



Supplementary figure S6 Network analysis of 3T3-L1 transcriptomes after USP7 knock down.

Ingenuity network explorer was used to detect for top scored biological interaction networks after USP7 knock down. Data are represented as described in Figure S3.

Chapter 3

MDM2 DEGRADES P53 BUT NOT TIP60 IN ADIPOGENESIS

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Manuscript in preparation

Abstract
Adipogenesis is regulated by a complex interplay of transcription factors, transcriptional cofactors and external stimuli. We recently reported the transcriptional cofactor Tat-interacting protein 60 (Tip60), a histone acetyltransferase, to be involved in adipogenesis. Tip60 protein levels are regulated by an ubiquitination-deubiquitination cycle in adipocyte differentiation: Tip60 protein is ubiquitinated and degraded in early adipogenesis, and stabilized upon deubiquitination during the later stages. While the deubiquitinase USP7 stabilizes Tip60 during adipogenesis, the E3 ubiquitin ligase responsible for Tip60 degradation in early adipogenesis was not identified yet. The E3 ubiquitin ligase murine double minute 2 (mdm2), an enzyme best known for its major role in regulating tumor suppressor p53 destruction, has been shown to ubiquitinate Tip60 in other cellular contexts, and is highly expressed in (pre)adipocytes. We therefore investigated whether mdm2 is responsible for Tip60 degradation in adipogenesis. In contrast to Tip60 protein, expression of the mdm2 protein, which displayed nuclear localization, as well as mRNA expression were relatively constant during 3T3-L1 adipogenesis. In mdm2-negative MEFs, Tip60 could still be ubiquitinated. In contrast, ubiquitination of p53 was completely dependent on re-introduction of mdm2 in these cells. Together, these findings indicate that E3 ubiquitin ligases other than mdm2 are probably responsible for Tip60 degradation during early adipogenesis.

Introduction

Reversible posttranslational modifications (PTM) present an important molecular mechanism to regulate complex biological processes like differentiation [1]. Ubiquitination, the covalent attachment of ubiquitin moieties to proteins, represents one such crucial PTM [2]. Proteins can be modified with a single ubiquitin molecule or with elongated polyubiquitin chains. Monoubiquitination has been implicated in many cellular processes, including membrane trafficking, histone function, transcription regulation, DNA repair, and DNA replication [3]. Polyubiquitination can target proteins for proteasomal degradation, but can also control non-proteolytic events, depending on the actual lysine residue within the ubiquitin molecule that is modified [4]. Ubiquitination requires a set of three enzymes, E1, E2 and E3. Substrate specificity is mainly defined by the various E2-E3 combinations possible [5]. While the large protein family of E2 ligases share many well-conserved catalytic domains [6], E3 ligases only share a few conserved motifs and are therefore very specific. There are ~600 putative E3s and substrate-recognition subunits of E3 complexes encoded in the human genome [7]. Protein ubiquitination can be reversed by deubiquitinases (DUBs), which are encoded by approximately 64 genes in the human genome [8].

One of the best-studied E3 ligases is mdm2, originally isolated from double minute extra-chromosomal elements discovered in a spontaneously transformed 3T3 cell line (3T3-DM) [9]. Increased mdm2 levels have been reported in both murine and human tumors (reviewed in [10,11]). It is a RING-finger E3 ubiquitin ligase that can specifically interact with and destabilize several proteins, including the tumor suppressor protein p53 [12,13,14] and the acetyltransferase Tip60 [15]. Interestingly, the deubiquitinase USP7 can stabilize Tip60 (Chapter 2), p53 [16] and mdm2 [17,18]. The balance between USP7 and mdm2 expression and/or activity will therefore determine the net effect on any common substrate, as USP7 could either directly stabilize substrate proteins or indirectly destabilize substrates through stabilization of mdm2.

Here we investigated the potential role of mdm2 in adipocyte differentiation for two reasons. First, mdm2 is highly expressed in differentiated adipocytes [19]. Secondly, we have recently shown that protein expression of Tip60, an established mdm2 substrate, is regulated by ubiquitination in adipogenesis [20] (Chapter 2). Our data

indicate that mdm2 serves as the dominant E3 ubiquitin ligase for p53, but not Tip60 in adipogenesis.

Results and Discussion

Mdm2 protein and mRNA levels remain constant during adipogenesis

To investigate the role of the E3 ubiquitin ligase mdm2 in adipogenesis, we first analyzed mdm2 protein levels at different time points during 3T3-L1 differentiation by Western blotting. Mdm2 protein levels were relatively constant during adipogenesis (Fig. 1A). Mdm2 mRNA levels are also relatively constant during adipocyte differentiation (Fig. 1B; data from [21]). We also examined the subcellular localization of mdm2 in mature adipocytes. Mdm2 displayed nuclear localization in differentiated 3T3-L1 adipocytes (Fig. 1C), the same subcellular localization as its potential substrate Tip60 (Chapter 2).

Mdm2 is not the only E3 ligase for Tip60

Expression of the Tip60 protein, but not mRNA, decreases during the first stages of 3T3-L1 differentiation [20], suggesting that this protein may be regulated by ubiquitination during the early steps of adipogenesis. We hypothesized that mdm2 may be the E3 ligase responsible for Tip60 ubiquitination during adipogenesis, because (i) mdm2 is highly expressed in differentiated adipocytes [19](Fig. 1a), mdm2 is localized in the nucleus (Fig. 1c) as is Tip60 (Chapter 2) and mdm2 can ubiquitinate Tip60, after overexpression of both proteins in cells and *in vitro* [15]. To investigate the relevance of mdm2 for Tip60 polyubiquitination we made use of a MEF cell line with double knockout of mdm2 and p53 (DKO cells). Tip60 was expressed together with histidine-tagged ubiquitin these cells, in the presence of absence of Mdm2. Cells were treated with proteasome inhibitor MG132 for 6 hours before harvesting. Purification of histidine-tagged proteins followed by Western blotting with an antibody directed against Tip60 revealed high molecular weight species of Tip60, indicative of polyubiquitination, showing that E3 ligases other than mdm2 can ubiquitinate the Tip60 protein (Fig. 2A). Reintroduction of mdm2 had little effect on Tip60 polyubiquitination, indicating that Mdm2 is not limiting for Tip60 ubiquitination. In contrast, p53 ubiquitination was nearly absent in DKO cells, and could be restored by reintroduction of mdm2. To corroborate these findings we performed knock down experiments in 3T3-L1 (pre)adipocytes. Knock down of mdm2 with various siRNA oligonucleotides did not result in significant inhibition of differentiation,

as assessed by triglyceride accumulation (data not shown). However, mdm2 knock down clearly increased p53 protein levels (Fig. 2B),

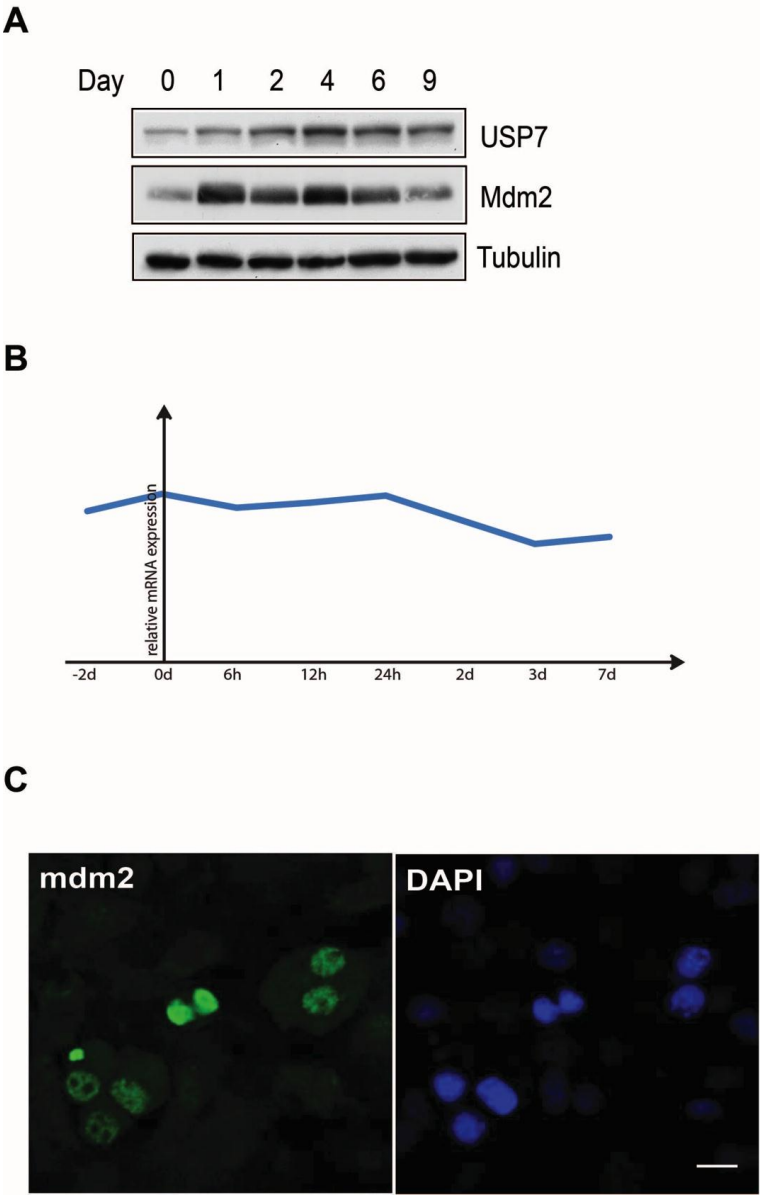

Fig. 1 Mdm2 expression in 3T3-L1 adipocyte differentiation. A, Mouse 3T3-L1 pre-adipocytes were differentiated into mature adipocytes and samples taken at different time points. USP7 protein level is analyzed with western blot. Tubulin is loaded as a control. B, Mdm2 mRNA expression during adipogenesis. Expression

profile was extracted from microarray data form Mikkelsen et al. [21]. C, Mdm2 is expressed in the nucleus of differentiated 3T3-L1 adipocytes. 3T3-L1 pre-adipocytes were differentiated into mature adipocytes and 5 days after differentiation cells were fixed and probed with Mdm2 antibody. Dapi is used as a nuclear staining.

supporting the view that mdm2 is important for p53 stabilization. Taken together, our findings indicate that mdm2 is the dominant E3 ligase for p53, but not Tip60.

Other E3 ligases expressed during adipocyte differentiation

Tip60 is an important transcriptional cofactor in adipogenesis, its protein level being tightly regulated by an ubiquitination-deubiquitination cycle [20](Chapter 2). While USP7 is the dominant deubiquitinase for Tip60 in adipogenesis (Chapter 2), our data indicate that the E3 ubiquitin ligase mdm2 is probably not responsible for Tip60 ubiquitination during early adipogenesis (Fig. 1 and 2). In addition, expression of Cul3, a second E3 ligase that was recently implicated in Tip60 ubiquitination [22], is very low in 3T3-L1 (pre)adipocytes [21]. We will now discuss other candidate E3 ligase(s) for Tip60 during adipogenesis. The first option is the p300 protein, which harbours intrinsic ubiquitin ligase activity [23], and has been shown to ubiquitinate Tip60 [24]. Expression of p300 is however constant during adipogenesis [25]. Alternatively, since the protein level of Tip60 is abruptly being down regulated at the beginning of adipogenesis and increases during the first days of adipogenesis [20], one may speculate that the expression of an E3 ligase responsible for Tip60 ubiquitination increases during the first day of differentiation and drops sharply after day 2 of differentiation. We analyzed mRNA expression levels of 250 RING finger/U-box E3 ubiquitin ligases [5] during differentiation in the gene expression data set from Mikkelsen et al. [21] and found 8 candidate E3 ubiquitin ligases that followed the expression pattern described above: Uhrf2, RNF207, Birc3, Trim2, Phf21a, Adnp, Mycbp2 and RNF103 (Fig. 3A and data not shown). This particular expression profile is not a common feature for E3 ligases in adipogenesis, as illustrated by the very different expression profiles of other E3 ligases (Fig. 3B). Furthermore, Uhrf2, Birc3, Phf21a and Adnp are predicted to localize to the nucleus, which would be a prerequisite for them to function as E3 ligases for the nuclear Tip60 protein. These 4 proteins therefore present interesting candidates to function as E3 ligases for

Tip60 in adipogenesis. The Phf21a gene, which encodes BHC80, a component of a BRAF35 histone deacetylase complex, is of special interest. Interestingly, we recently identified this protein in a RNAi-based screen as being essential for differentiation of the human SGBS (pre)adipocyte cell line [26]. It should be noted however that this protein, together with the Adnp protein, was classified as E3 ubiquitin ligases based on sequence homology and its enzymatic activity has not been proven yet [5]. In addition, our selection of candidate E3 ligases is based on mRNA expression, which may not reliably reflect enzymatic activity of E3 ligases during adipocyte differentiation. Whether and how Uhrf2, Birc3, Phf21a and Adnp (and other E3 ligases) are involved in Tip60 regulation and adipogenesis therefore needs further prove.

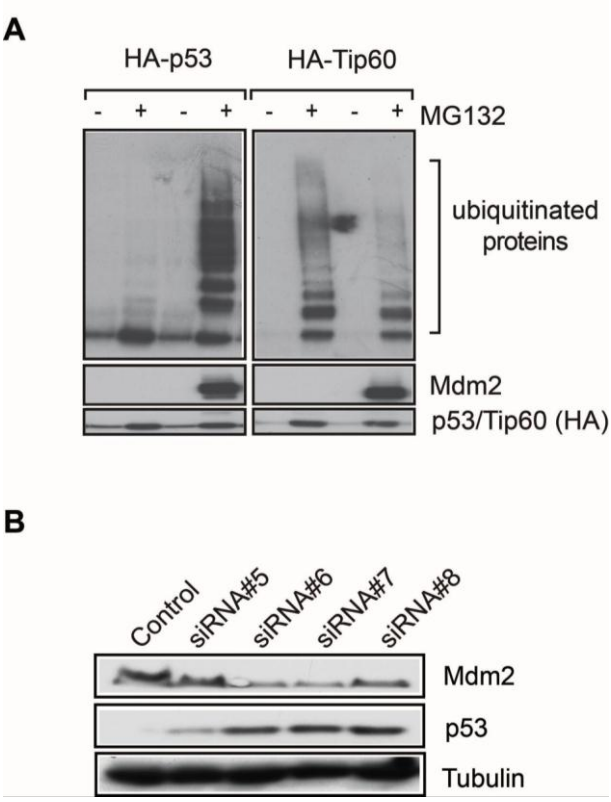

Fig. 2 Mdm2 is the dominant E3 ligase for p53, but not Tip60. A, MEFs with DKO Mdm2/p53 is transfected with p53 or Tip60 plasmid and his-ubi expression factor. B, Knockdown of Mdm2 during adipogenesis of 3T3-L1 cells. Cells were

harvested 6 days after differentiation and Mdm2 and p53 protein levels were analyzed by Western blotting.

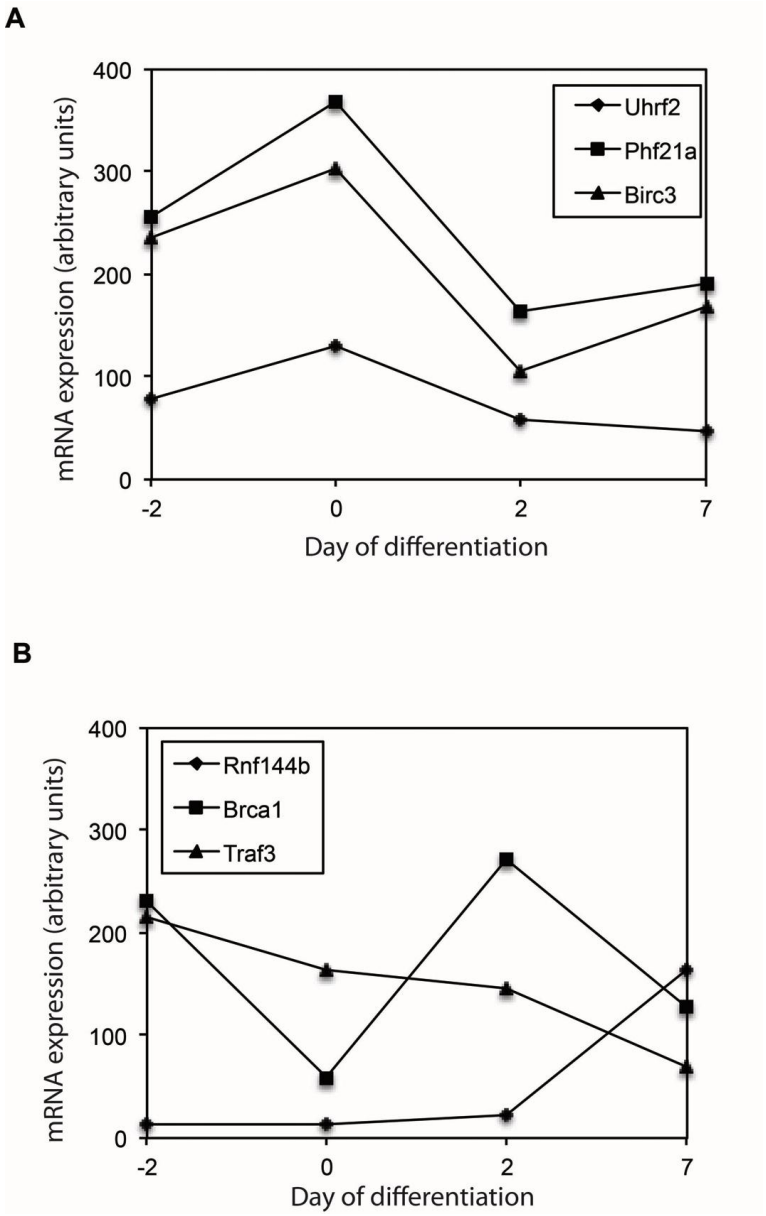

Fig. 3 mRNA expression of selected E3 ubiquitin ligases during 3T3-L1 adipocyte differentiation. A, Examples of E3 ligases with highest mRNA expression around

day 0 of differentiation. B, Examples of E3 ligases with highest expression levels at time points other than day 0. Expression profiles were extracted from microarray data from Mikkelsen et al. [21].

In summary, we have shown here that Mdm2 is expressed in the nucleus of adipocytes where it apparently regulates p53 but not Tip60 levels. How its substrate specificity is regulated during the differentiation process is of interest and needs further study.

Materials and methods

Cell culture

Mdm2/p53 DKO mouse embryonic fibroblasts, a gift of Dr. Gigi Lozano, were maintained in DMEM Glutamax (Dulbecco) containing 10% foetal calf serum (Gibco Life Technologies), penicillin and streptomycin (100mg/ml each, Gibco Life Technologies). The 3T3-L1 cell line was cultured in the same media but with 10% bovine serum (Gibco Life Technologies).

Differentiation assays and western blot analysis

3T3-L1 cells were grown to confluence and after two days incubated with culture medium containing dexamethasone (250nM), IBMX (500uM), insulin (170nM) for two days. At day 3, medium was changed for culture medium supplemented with insulin (170nM) and left for a week or harvested at different days. Transfection with siRNA oligonucleotides was performed as described [20,27], using ON-TARGET plus siRNA J-041098-00-0002 (mdm2) and D-001210-01-20 (control).

Western blot analysis was performed as described [20,28], using the following antibodies: anti-mdm2 (4B2), anti-p53 (DO-1; sc-126) from SantaCruz Biotechnology (SantaCruz, CA) and 1C12 from Cell Signaling Technology (Danvers, MA); anti-tubulin (T9026) and anti-HA (H9658) from Sigma-Aldrich (St. Louis, MO); anti-mouse-HRP (1858413) from Pierce Biotechnologies (Rockford, IL).

Immunofluorescence

For immunofluorescence staining, 3T3-L1 cells were plated on glass coverslips. Cells were differentiated for 5 days and fixed with 4% paraformaldehyde for 20 min RT and permeabilized in PBS supplemented with 0.5% Triton for 10 min. After 30-min incubation in blocking buffer, cells were stained with primary antibodies for 2h at room temperature, and then incubated with secondary fluorochrome-conjugated antibodies. After several washes, coverslips were mounted in Dabco-DAPI and analyzed with a microscope.

Transfection and ubiquitination assays

MEF cells with mdm2/p53 DKO were transfected with HA-p53 or HA-Tip60 plasmid, in presence of absence of Mdm2 expression plasmids, together with his6-ubi constructs. Twenty-four hours after transfection,

20 nM MG132 (Biomol) was added for 6h. Lysis and purification on Ni-NTA beads was performed as described [29]. After washing, beads were boiled and subjected to western blot analysis with HA antibody. Input samples were subjected to western blot with HA antibody and Mdm2 antibody.

References

1. Walsh CT, Garneau-Tsodikova S, Gatto GJ, Jr. (2005) Protein posttranslational modifications: the chemistry of proteome diversifications. AngewChemIntEd Engl 44: 7342-7372.
2. Kerscher O, Felberbaum R, Hochstrasser M (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. Annual review of cell and developmental biology 22: 159-180.
3. Sigismund S, Polo S, Di Fiore PP (2004) Signaling through monoubiquitination. Current topics in microbiology and immunology 286: 149-185.
4. Sun L, Chen ZJ (2004) The novel functions of ubiquitination in signaling. Current opinion in cell biology 16: 119-126.
5. van Wijk SJ, de Vries SJ, Kemmeren P, Huang A, Boelens R, et al. (2009) A comprehensive framework of E2-RING E3 interactions of the human ubiquitin-proteasome system. Molecular systems biology 5: 295.
6. van Wijk SJ, Timmers HT (2010) The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 24: 981-993.
7. Li W, Bengtson MH, Ulbrich A, Matsuda A, Reddy VA, et al. (2008) Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. PloS one 3: e1487.
8. Nijman SMB, Luna-Vargas MPA, Velds A, Brummelkamp TR, Dirac AMG, et al. (2005) A genomic and functional inventory of deubiquitinating enzymes. Cell 123: 773-786.
9. Fakharzadeh SS, Trusko SP, George DL (1991) Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. The EMBO journal 10: 1565-1569.
10. Juven-Gershon T, Oren M (1999) Mdm2: the ups and downs. Molecular medicine 5: 71-83.
11. Momand J, Wu HH, Dasgupta G (2000) MDM2--master regulator of the p53 tumor suppressor protein. Gene 242: 15-29.
12. Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. Nature 387: 296-299.
13. Honda R, Tanaka H, Yasuda H (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS letters 420: 25-27.
14. Kubbutat MH, Jones SN, Vousden KH (1997) Regulation of p53 stability by Mdm2. Nature 387: 299-303.

15. Legube G, Linares LK, Lemercier C, Scheffner M, Khochbin S, et al. (2002) Tip60 is targeted to proteasome-mediated degradation by Mdm2 and accumulates after UV irradiation. EMBO J 21: 1704-1712.
16. Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, et al. (2002) Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature 416: 648-653.
17. Li M, Brooks CL, Kon N, Gu W (2004) A dynamic role of HAUSP in the p53-Mdm2 pathway. Molecular cell 13: 879-886.
18. Cummins JM, Vogelstein B (2004) HAUSP is required for p53 destabilization. Cell Cycle 3: 689-692.
19. Berberich SJ, Litteral V, Mayo LD, Tabesh D, Morris D (1999) mdm-2 gene amplification in 3T3-L1 preadipocytes. Differentiation 64: 205-212.
20. van Beekum O, Brenkman AB, Grontved L, Hamers N, van den Broek NJ, et al. (2008) The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARgamma. Endocrinology 149: 1840-1849.
21. Mikkelsen TS, Xu Z, Zhang X, Wang L, Gimble JM, et al. (2010) Comparative epigenomic analysis of murine and human adipogenesis. Cell 143: 156-169.
22. Bhoumik A, Singha N, O'Connell MJ, Ronai ZA (2008) Regulation of TIP60 by ATF2 modulates ATM activation. The Journal of biological chemistry 283: 17605-17614.
23. Grossman SR, Deato ME, Brignone C, Chan HM, Kung AL, et al. (2003) Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science 300: 342-344.
24. Col E, Caron C, Chable-Bessia C, Legube G, Gazzeri S, et al. (2005) HIV-1 Tat targets Tip60 to impair the apoptotic cell response to genotoxic stresses. EMBO J 24: 2634-2645.
25. Takahashi N, Kawada T, Yamamoto T, Goto T, Taimatsu A, et al. (2002) Overexpression and ribozyme-mediated targeting of transcriptional coactivators CREB-binding protein and p300 revealed their indispensable roles in adipocyte differentiation through the regulation of peroxisome proliferator-activated receptor gamma. J Biol Chem 277: 16906-16912.
26. Verrijn Stuart AA, Schipper HS, Tasdelen I, Egan DA, Prakken BJ, et al. (2012) Altered plasma adipokine levels and in vitro adipocyte differentiation in pediatric type 1 diabetes. The Journal of clinical endocrinology and metabolism 97: 463-472.

27. Koppen A, Houtman R, Pijnenburg D, Jeninga EH, Ruijtenbeek R, et al. (2009) Nuclear receptor-coregulator interaction profiling identifies TRIP3 as a novel PPARgamma cofactor. MolCell Proteomics 8: 2212-2226.
28. Jeninga EH, van Beekum O, van Dijk AD, Hamers N, Hendriks-Stegeman BI, et al. (2007) Impaired peroxisome proliferator-activated receptor gamma function through mutation of a conserved salt bridge (R425C) in familial partial lipodystrophy. MolEndocrinol 21: 1049-1065.
29. Stad R, Little NA, Xirodimas DP, Frenk R, Van Der Eb AJ, et al. (2001) Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. EMBO Rep 2: 1029-1034.

Chapter 4

ALLELE COMPENSATION IN TIP60+/- MICE RESCUES WHITE ADIPOSE TISSUE FUNCTION *IN VIVO*

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Submitted

Abstract
Adipose tissue is a key regulator of energy homeostasis. The amount of adipose tissue is largely determined by adipocyte differentiation (adipogenesis), a process that is regulated by the concerted actions of multiple transcription factors and cofactors. Based on *in vitro* studies in murine 3T3-L1 preadipocytes and human primary preadipocytes, the transcriptional cofactor and acetyltransferase Tip60 was recently identified as an essential adipogenic factor. We therefore investigated the role of Tip60 on adipocyte differentiation and function, and possible consequences on energy homeostasis, *in vivo*. As homozygous inactivation results in early embryonic lethality, Tip60+/- mice were used. Heterozygous inactivation of Tip60 had no effect on body weight, despite higher food intake by Tip60+/- mice. No major effects of heterozygous inactivation of Tip60 were observed on adipose tissue and liver, and Tip60+/- displayed normal glucose tolerance, both on a low fat and a high fat diet. While Tip60 mRNA was reduced to 50% in adipose tissue, the protein levels were unaltered, suggesting compensation by the intact allele. These findings indicate that the *in vivo* role of Tip60 in adipocyte differentiation and function cannot be properly addressed in Tip60+/- mice, but requires the generation of adipose tissue-specific knock out animals or specific knock-in mice.

Introduction

The relationship between obesity and its complications, such as type 2 diabetes and cardiovascular diseases, has firmly established adipose tissue as a key regulator of glucose and lipid metabolism [1]. Adipose tissue regulates metabolism through at least two different mechanisms: the storage and release of lipids, and the secretion of so-called adipokines, which function in an endocrine or paracrine fashion. Expansion of adipose tissue, as seen in obese individuals, not only affects the storage of lipids as triglycerides in lipid droplets, but also results in qualitative and quantitative changes in a number of adipokines [2]. The amount of mature adipocytes is largely determined by the differentiation of fibroblast-like mesenchymal stem cells into adipocytes, a process called adipogenesis [1,3]. Adipogenesis is regulated by a cascade of transcription factors, ultimately leading to the induction of the transcription factor Peroxisome proliferator activator receptor γ (PPARγ) [1,4]. In general, adipogenic transcription factors activate transcription of target genes in concert and in association with coregulatory proteins, a class of proteins that do not bind to DNA themselves. The route from target gene binding by a transcription factor to gene transcription is a multistep process, which involves both recruitment and release of coregulators, chromatin remodelling and activation of the basal transcription machinery. Some coregulators, such as Tat-interactive protein-60KDa (Tip60), can alter the local chromatin context. Tip60 is a member of the MYST family of histone acetyltransferases, named after its founding members MOZ, Ybf2/Sas3, Sas2 and Tip60, which share a highly conserved MYST acetyltransferase domain, but display limited homology outside this region [5,6]. Tip60 is the catalytic subunit of the highly conserved NuA4 acetyltransferase complex [7,8], which plays a key role in transcription regulation, cell cycle and checkpoint control, apoptosis and DNA damage repair [9,10,11]. Tip60 can be recruited to the promoter of certain genes through transient interaction with a variety of different transcription factors, where it can acetylate histone proteins (H4, H2A, H2A.X and H2A.Z) and various transcription factors, thereby activating or repressing transcription [10]. Activation requires the HAT activity of Tip60, while repression is thought to be independent from its HAT activity and may result from its interaction with transcriptional silencers and histone deacetylases [10]. Tip60 transiently associates with a growing list of specific transcription factors where it acts either

as a coactivator or as a corepressor. Recently, we identified Tip60 as a positive regulator of PPARγ transcriptional activity [12]. Tip60 targets the AF1 region of PPARγ, a region of the protein implicated in isotype-selective gene expression and adipogenesis [13,14]. Chromatin immunoprecipitation experiments showed that the endogenous Tip60 protein is recruited to PPARγ target genes in mature 3T3-L1 adipocytes, but not in pre-adipocytes, indicating that Tip60 requires PPARγ for its recruitment to PPARγ target genes [12]. Interestingly, expression of the Tip60 protein, but not mRNA, increases during the first stages of 3T3-L1 differentiation [12], suggesting that regulation of Tip60 protein levels may play an important role in early adipogenesis. Indeed, transcriptome analysis revealed several cell cycle genes to be regulated by Tip60, and knock down of Tip60 resulted in impaired mitotic clonal expansion, an early step in adipogenesis (Chapter 2). Together, these findings qualify the MYST acetyltransferase Tip60 as an adipogenic factor, that operates through two different mechanisms: in early adipogenesis it regulates several cell cycle genes during MCE, while it functions as a PPARγ coactivator during the later stages of adipocyte differentiation. A role for Tip60 in adipocyte differentiation and/or function *in vivo* has however not been established so far. Here, we investigated the metabolic role of Tip60 *in vivo*, making use of heterozygous Tip60 knock out mice. Our data indicate that while heterozygous loss of Tip60 affects some metabolic parameters (caloric intake) and metabolic organs (liver weight), Tip60+/- mice display largely unaltered glucose metabolism.

Materials and Methods

Materials

The following antibodies were used: anti-Tip60 (sc-5725), Santa Cruz Biotechnologies; anti-tubulin (ab6046), Abcam; anti-rabbit-HRP (111035144) and anti-mouse-HRP (115035146), Jackson Immunoresearch Laboratories Inc.

Animal studies.

WT C57BL/6J mice (8 weeks; Charles River Laboratories) and Tip60$^{+/-}$ mice [11,15] that had been backcrossed to C57BL/6J for 12 generations, were age-matched and fed standard chow until age 11 weeks, and subsequently fed LFD (10 kcal% fat, Research Diet D12450B) or HFD (45 kcal% fat, Research Diet D12451) for 19 weeks. For the intraperitoneal glucose tolerance test (IP-GTT), mice (age 28 weeks) were fasted overnight, glucose was injected intraperitoneally (0.5g/kg body weight) and blood glucose levels were measured before, and at multiple time points after glucose injection (Accu-chek, Roche). All mouse study protocols were approved by the Utrecht University Ethical Committee for Animal Experimentation (protocol 2010.III.01.008) and were in accordance with current Dutch laws on animal experimentation.

AT and liver immunohistochemistry and liver triglycerides

Hematoxylin and eosin (H&E) staining of AT and liver sections was performed using standard protocols. Liver triglycerides were determined in liver homogenates prepared in buffer containing 250mM sucrose, 1mM EDTA, and 10mM Tris-HCl at pH 7.5 using a commercially available kit (Instruchemie, Delfzijl, The Netherlands) according to the manufacturer's instructions.

RNA extraction, quantitative PCR and Western blot analysis.

Snap-frozen epididymal adipose tissue was homogenized and RNA was extracted using Trizol (Invitrogen). RNA was purified on an RNeasy micro column (Qiagen), RNA integrity was checked with a Bioanalyzer (Agilent), and cDNA synthesis was performed with iScript (Bio-Rad). Quantitative PCR with SYBR Green (Bio-Rad) were run on a MyIq machine (Bio-Rad). Primers for quantitative RT-PCR were designed with the universal probe library (Roche) sequences were as follows: mTip60 Forw GCTGCTTATTGAGTTCAGCTATG; mTip60 Rev GGATCTCCAAGATGGTTTGG; m36B4 Forw

AGCGCGTCCTGGCATTGTGTGG; m36B4 Rev GGGCAGCAGTGGTGGCAGCAGC.

For protein analysis, eWAT was lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Etten-Leur, The Netherlands) and proteins were analyzed by Western blotting as described [12,16].

Results

Tip60+/- mice have normal body weight despite higher food intake

As homozygous Tip60 null mice (Tip60-/-) are embryonic lethal around the blastocyst stage [11,15], we used heterozygous Tip60 KO mice to investigate the metabolic role of Tip60. Tip60+/- mice, originally generated by replacing exons 1-9 with a neomycin-targeting vector and maintained on a 129sv- C57BL/6 mixed genetic background[11,15], were first backcrossed to a C57BL/6 genetic background.

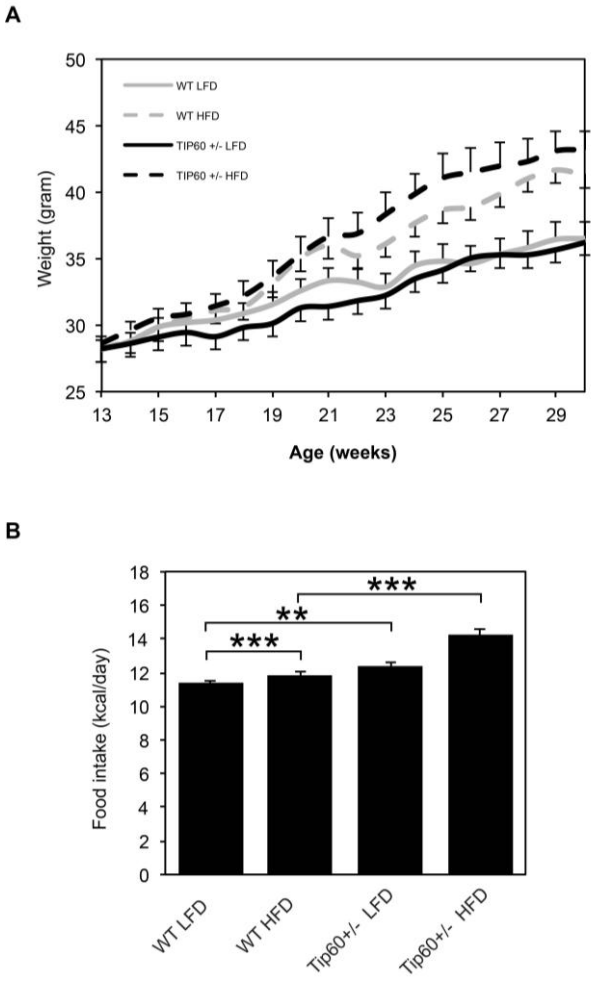

Figure 1. Tip60+/- mice display normal bodyweight with higher daily caloric intake. A, WT and Tip60+/- mice after were weighed each week during 18 weeks of LFD or HFD feeding. Error bars represent means ± s.e.m. N=6-8 mice per group,

total 30 mice. B, Daily food intake of WT and Tip60+/- mice on LFD and HFD regimens. ** $p<0.01$, *** $p<0.001$.

WT and Tip60+/- mice were fed a low fat diet (10 kcal% fat; LFD) or high fat diet (45 kcal% fat; HFD) for 19 weeks. While WT and Tip60+/- mice on HFD displayed significantly higher body weights compared to the LFD groups, no significant difference was observed between the genotypes (Fig. 1A). When caloric intake was analyzed, significant differences were observed between the 4 groups, with Tip60+/- on HFD having the largest daily caloric intake (Fig. 1B). Taken together, these findings indicate that heterozygous Tip60 deletion may affect appetite (daily caloric intake) without affecting weight gain in a 19 weeks HFD regime.

Heterozygous Tip60 deletion affects liver weight but not insulin sensitivity

As knock down of Tip60 results in impaired adipogenesis [12,17], we analyzed the epipdydimal WAT (eWAT) depot of Tip60+/- mice on LFD and HFD. As shown in Fig. 2A, WT and Tip60+/- mice on HFD displayed significantly larger eWAT depots compared to the LFD groups, but no significant difference was observed between the genotypes. Circulating FFA levels were not altered by diet or genotype (Fig. 2B). AT morphology was also clearly affected by HFD, with larger adipocytes, but not by genotype (Fig. 2C).

When the liver weights were analyzed, significant differences were observed between the LFD and HFD groups, but not between WT and Tip60+/- animals (Fig. 3A). Liver triglyceride (TG) content tended to be higher in HFD groups, but was independent of genotype (Fig. 3B). HFD feeding resulted in the formation of lipid droplets in the liver, but no additional effects were observed by heterozygous Tip60 inactivation (Fig. 3C).

Next we analyzed whether glucose metabolism was affected by heterozygous deletion of Tip60. Glucose tolerance measured via an intraperitoneal glucose tolerance test (IP-GTT) was clearly impaired by HFD in WT and Tip60+/- mice, but no difference between the genotypes was observed (Fig. 3A, B). In summary, these results show that heterozygous inactivation of Tip60 has no dramatic effects on adipose tissue or liver, nor does it alter glucose tolerance.

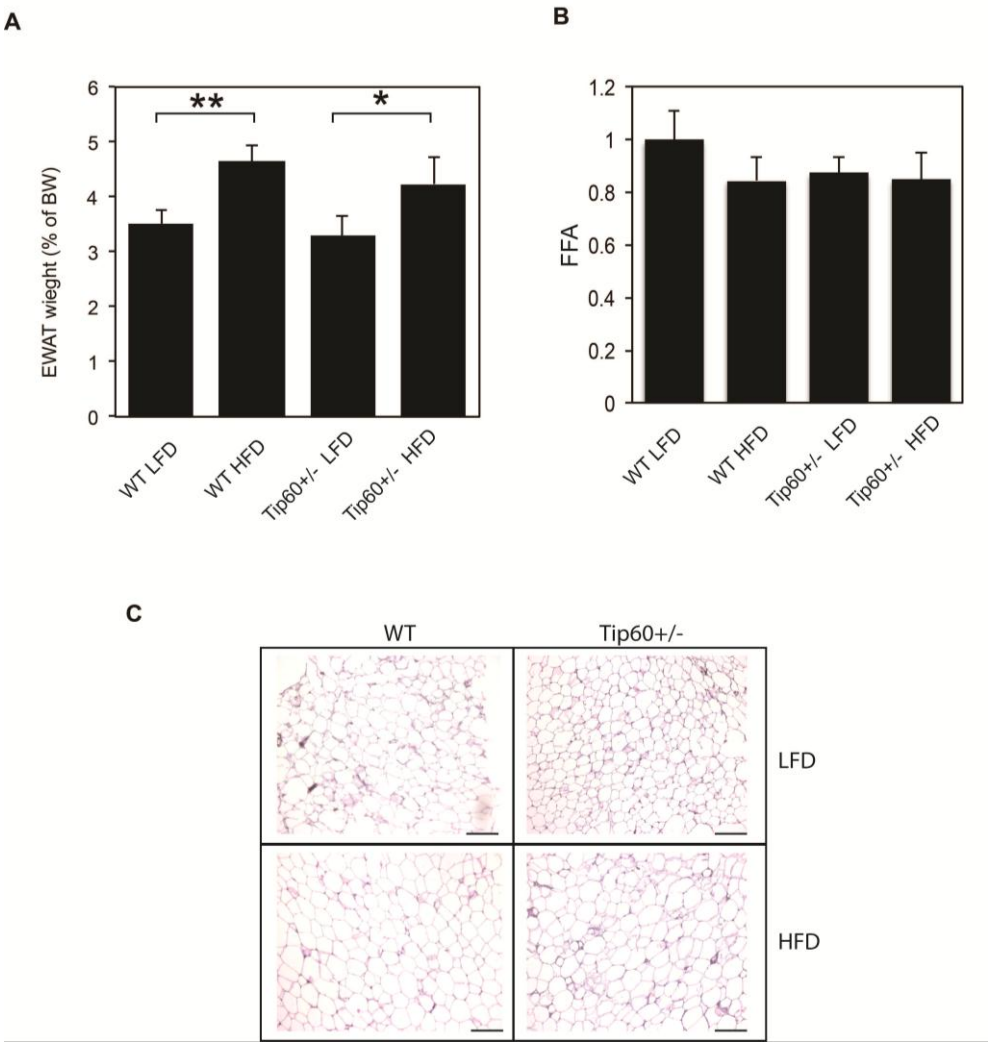

Figure 2. Tip60+/- mice display normal eWAT weight and morphology. A, Epididymal fat pad (eWAT) weights of WT and Tip60+/- mice on a LFD and HFD regimen, measured after termination. * $p<0.05$, ** $p<0.01$. B, Plasma free fatty acid (FFA) levels. N=6-8 mice per group, total 30 mice. C, H&E staining of representative eWAT sections from wt and Tip60+/- mice after 18 weeks of LFD feeding. Scale bars indicate 100 m.

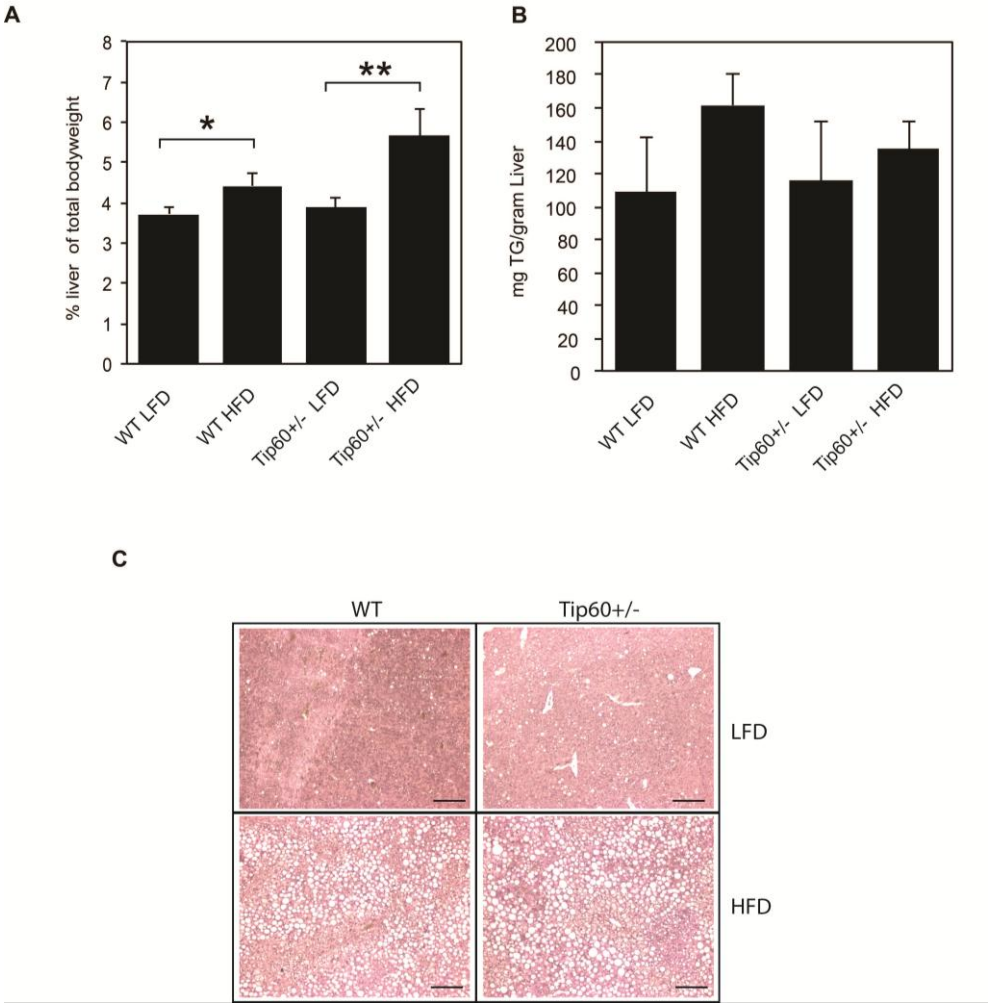

Figure 3. Tip60+/- mice display normal liver weight, TG content and morphology. A, Liver weight as percentage of total body weight of WT and Tip60+/- mice on a LFD and HFD regimen, measured after termination. * $p<0.05$, ** $p<0.01$. B, Liver triglyceride (TG) content. N=6-8 mice per group, total 30 mice. C, H&E staining of representative liver sections of the WT and Tip60+/- mice fed LFD or HFD for 19 weeks. Scale bars indicate 100 μm.

Reduced Tip60 mRNA but not protein expression in eWAT of Tip60$^{+/-}$ mice

As knock down of Tip60 results in impaired adipogenesis in murine 3T3-L1 preadipocytes [12] and human primary preadipocytes [17], but

no effect was observed of heterozygous Tip60 deletion on total body weight and eWAT depot weight (Fig. 2A), we examined Tip60 mRNA and protein levels in WT and Tip60+/- mice. As expected, a 50% reduction of Tip60 mRNA levels was observed in eWAT of Tip60+/- mice compared to WT controls (Fig. 5A). However, when Tip60 protein was analyzed by Western blotting, no significant difference was observed between WT and Tip60+/- mice (Fig. 5B). These findings suggest that at least in eWAT the intact Tip60 allele probably compensates for the heterozygous loss of Tip60. Taken together, our data indicate that while heterozygous inactivation of Tip60 affects food intake (Fig. 1B), Tip60+/- mice display largely unaltered overall glucose metabolism (Fig. 4). Heterozygous inactivation of Tip60 did not affect eWAT weight or histology (Fig. 2), which may be explained by compensation by the intact allele (Fig. 5).

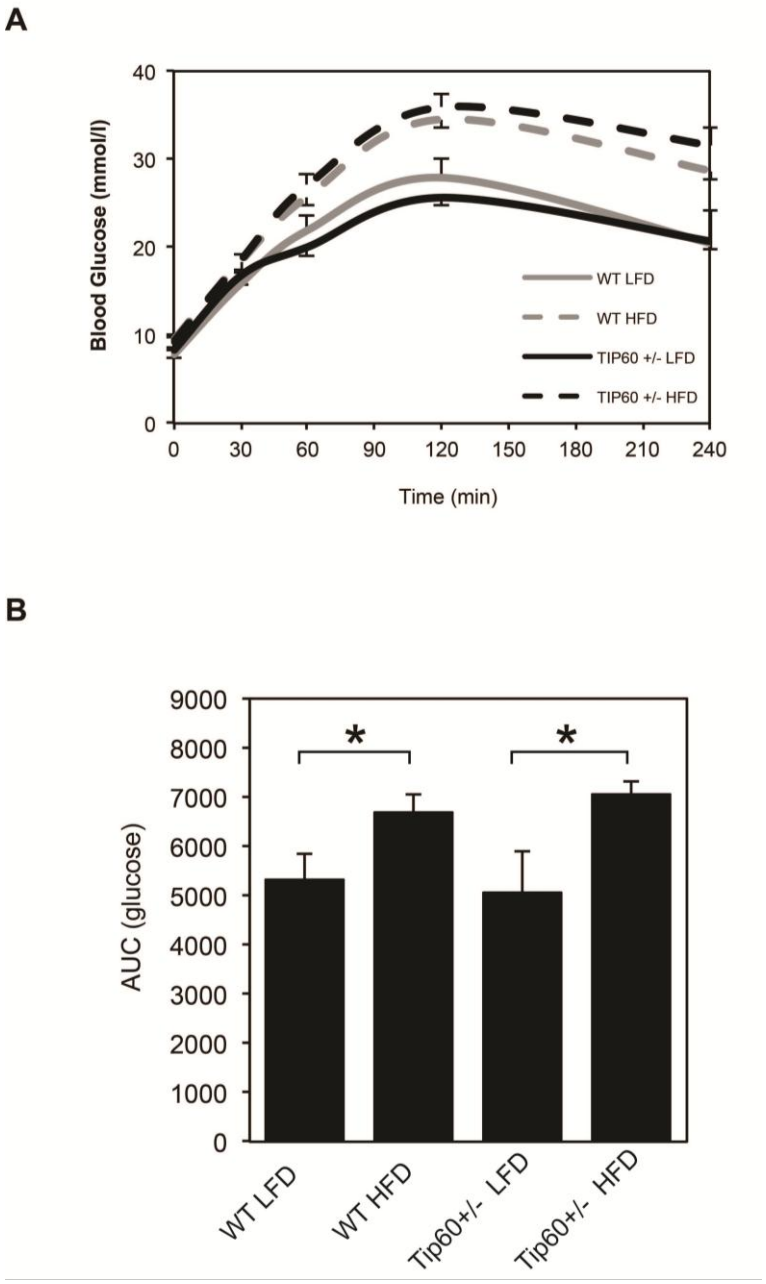

Figure 4. Tip60+/- mice display normal glucose tolerance. A,B, Intra-peritoneal glucose tolerance test was performed in WT and Tip60+/- mice after 18 weeks of LFD or HFD. Plasma glucose concentrations (A) and the area under the curve (B) for the various groups are shown. * $p<0.05$.

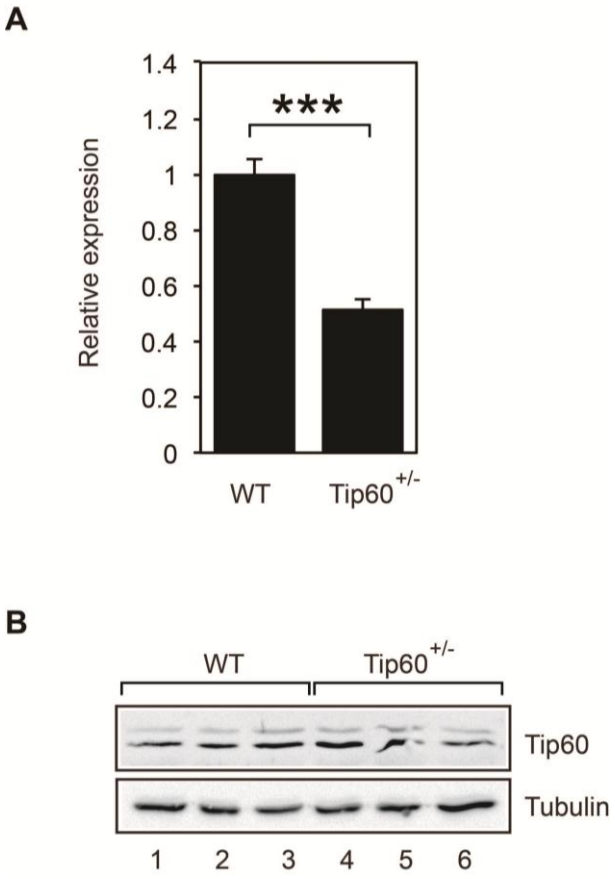

Figure 5. Tip60 mRNA, but not protein, is reduced in eWAT of Tip60+/- mice. A, Tip60 mRNA expression in eWAT of WT and Tip60+/- mice as determined by quantitative RT-PCR (N=5 per group). Mean expression in WT mice was set at 1. *** $p<0.001$. B, Tip60 protein expression in eWAT of WT and Tip60+/- mice as determined by Western blotting. Tubulin was used as a loading control.

Discussion

Based on *in vitro* studies in murine 3T3-L1 preadipocytes [12] and human primary preadipocytes [17], the transcriptional coativator and acetyltransferase Tip60 was identified as an essential adipogenic factor. We therefore investigated the role of Tip60 on adipocyte differentiation and function, and possible consequences on energy homeostasis, *in vivo*. As homozygous deletion of Tip60 results in embryonic lethality [11,15], we made use of heterozygous knock out animals. We observed no significant differences in AT weight and morphology between Tip60+/- animals and WT controls, either under normal feeding conditions (LFD) or upon 19 weeks HFD challenge. Glucose tolerance (IP-GTT) and lipid metabolism (plasma FFA, liver TG content) were also not different between the groups, indicating that heterozygous deletion of Tip60 has no major effects on AT development and function. Unfortunately, while Tip60 mRNA expression levels were reduced by approximately 50% in eWAT of Tip60+/- animals, Tip60 protein expression levels were not significantly different between the two genotypes (Fig. 5). Also in other tissues steady-state levels of Tip60 protein were unaltered in Tip60+/- mice [18], suggesting that the intact allele can compensate for the heterozygous Tip60 inactivation at least in some tissues. These observations preclude any conclusion on the role of Tip60 in AT *in vivo* at present. Next to Tip60, several other transcriptional cofactors have been implicated in energy homeostasis, some of which play a role in adipocyte differentiation and/or function, including the coactivators SRC12, -2 and -3, CBP and p300, and the corepressors RIP140, NCoR and SMRT [19,20]. The role of some of these factors in adipocyte differentiation and/or function has been studied *in vivo* (e.g. SRC1 and -2; [21], RIP140 [22]), but, like Tip60, whole body gene knock outs of several cofactor genes results in embryonic lethality (e.g. NCoR[23]; SMRT[24]; CBP and p300 [25]). Alternative approaches have therefore been used, including tissue-specific homozygous gene inactivation (e.g. [26]), or generation of animals bearing specific knockin mutations [27,28,29,30]. Similar approaches are required to establish the role of Tip60 in AT development and function, and its subsequent role in energy homeostasis, *in vivo*.

Recently, Tip60 was implicated in gluconeogenesis [31]. Using yeast proteome microarrays, Lin *et al.* found the NuA4 complex to acetylate the yeast phosphoenolpyruvate carboxykinase enzyme (Pck1p), a rate-limiting enzyme in gluconeogenesis. Acetylation of Pck1p was crucial

for enzymatic activity and the ability to grow on non-fermentable carbon sources [31]. In addition, Tip60-mediated acetylation of the mammalian homologue PEPCK may also be important for gluconeogenesis, as knock down of Tip60 in the human hepatoma cell line HepG2 resulted in reduced glucose secretion [31]. In our *in vivo* studies fasting glucose levels unaltered in Tip60+/- mice (data not shown). It should be noted however that we did not specifically address hepatic gluconeogenesis. Furthermore, Tip60 protein expression levels in the liver may also be only marginally affected in the liver, analogous to our observations in eWAT (Fig. 5). Therefore, additional studies are required to address the potential role of Tip60 in gluconeogenesis *in vivo*.

Despite the fact that steady-state levels of Tip60 protein were unaltered in eWAT and several other tissues in Tip60+/- mice (Fig. 5;[18]), these animals display accelerated onset and enhanced penetrance of Myc-induced B-cell lymphomas when crossed with E_{μ}-*myc* transgenic animals [11]. This phenotype suggests that the intact Tip60 allele does not compensate for heterozygous inactivation of Tip60 in all cell types and tissues. Also in our study Tip60+/- animals displayed a phenotypic alteration: heterozygous inactivation of Tip60 resulted in higher daily caloric intake. The higher caloric intake did not result in increased body weight, suggesting that energy expenditure was increased. Future studies are required to establish whether this may be explained by reduced Tip60 protein levels in metabolic organs other than eWAT (i.e. brain, BAT) in Tip60+/- animals.

References

1. Rosen ED, Spiegelman BM (2006) Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444: 847-853.
2. Guilherme A, Virbasius JV, Puri V, Czech MP (2008) Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. NatRevMolCell Biol 9: 367-377.
3. Lefterova MI, Lazar MA (2009) New developments in adipogenesis. Trends EndocrinolMetab 20: 107-114.
4. Farmer SR (2006) Transcriptional control of adipocyte formation. Cell Metab 4: 263-273.
5. Yang XJ (2004) The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. Nucleic Acids Res 32: 959-976.
6. Utley RT, Cote J (2003) The MYST family of histone acetyltransferases. CurrTopMicrobiolImmunol 274: 203-236.
7. Doyon Y, Cayrou C, Ullah M, Landry AJ, Cote V, et al. (2006) ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. MolCell 21: 51-64.
8. Doyon Y, Cote J (2004) The highly conserved and multifunctional NuA4 HAT complex. CurrOpinGenetDev 14: 147-154.
9. Squatrito M, Gorrini C, Amati B (2006) Tip60 in DNA damage response and growth control: many tricks in one HAT. Trends Cell Biol 16: 433-442.
10. Sapountzi V, Logan IR, Robson CN (2006) Cellular functions of TIP60. IntJBiochemCell Biol 38: 1496-1509.
11. Gorrini C, Squatrito M, Luise C, Syed N, Perna D, et al. (2007) Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature 448: 1063-1067.
12. van Beekum O, Brenkman AB, Grontved L, Hamers N, van den Broek NJ, et al. (2008) The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARgamma. Endocrinology 149: 1840-1849.
13. Hummasti S, Tontonoz P (2006) The peroxisome proliferator-activated receptor N-terminal domain controls isotype-selective gene expression and adipogenesis. MolEndocrinol 20: 1261-1275.
14. Bugge A, Grontved L, Aagaard MM, Borup R, Mandrup S (2009) The PPARgamma2 A/B-domain plays a gene-specific role in

transactivation and cofactor recruitment. Molecular endocrinology 23: 794-808.
15. Hu Y, Fisher JB, Koprowski S, McAllister D, Kim MS, et al. (2009) Homozygous disruption of the Tip60 gene causes early embryonic lethality. DevDyn 238: 2912-2921.
16. Jeninga EH, van Beekum O, van Dijk AD, Hamers N, Hendriks-Stegeman BI, et al. (2007) Impaired peroxisome proliferator-activated receptor gamma function through mutation of a conserved salt bridge (R425C) in familial partial lipodystrophy. MolEndocrinol 21: 1049-1065.
17. Gronniger E, Wessel S, Kuhn SC, Sohle J, Wenck H, et al. (2010) A new protocol for functional analysis of adipogenesis using reverse transfection technology and time-lapse video microscopy. Cell BiolInt 34: 737-746.
18. Fisher JB, Kim MS, Blinka S, Ge ZD, Wan T, et al. (2012) Stress-induced cell-cycle activation in Tip60 haploinsufficient adult cardiomyocytes. PloS one 7: e31569.
19. Koppen A, Kalkhoven E (2010) Brown vs white adipocytes: The PPARgamma coregulator story. FEBS Lett 584: 3250-3259.
20. Feige JN, Auwerx J (2007) Transcriptional coregulators in the control of energy homeostasis. Trends Cell Biol 17: 292-301.
21. Picard F, Gehin M, Annicotte J, Rocchi S, Champy MF, et al. (2002) SRC-1 and TIF2 control energy balance between white and brown adipose tissues. Cell 111: 931-941.
22. Leonardsson G, Steel JH, Christian M, Pocock V, Milligan S, et al. (2004) Nuclear receptor corepressor RIP140 regulates fat accumulation. Proceedings of the National Academy of Sciences of the United States of America 101: 8437-8442.
23. Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, et al. (2000) Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell 102: 753-763.
24. Jepsen K, Solum D, Zhou T, McEvilly RJ, Kim HJ, et al. (2007) SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. Nature 450: 415-419.
25. Yao TP, Oh SP, Fuchs M, Zhou ND, Ch'ng LE, et al. (1998) Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell 93: 361-372.

26. Li P, Fan W, Xu J, Lu M, Yamamoto H, et al. (2011) Adipocyte NCoR knockout decreases PPARgamma phosphorylation and enhances PPARgamma activity and insulin sensitivity. Cell 147: 815-826.
27. Nofsinger RR, Li P, Hong SH, Jonker JW, Barish GD, et al. (2008) SMRT repression of nuclear receptors controls the adipogenic set point and metabolic homeostasis. Proceedings of the National Academy of Sciences of the United States of America 105: 20021-20026.
28. Shikama N, Lutz W, Kretzschmar R, Sauter N, Roth JF, et al. (2003) Essential function of p300 acetyltransferase activity in heart, lung and small intestine formation. EMBO J 22: 5175-5185.
29. Yamamoto H, Williams EG, Mouchiroud L, Canto C, Fan W, et al. (2011) NCoR1 is a conserved physiological modulator of muscle mass and oxidative function. Cell 147: 827-839.
30. Roth JF, Shikama N, Henzen C, Desbaillets I, Lutz W, et al. (2003) Differential role of p300 and CBP acetyltransferase during myogenesis: p300 acts upstream of MyoD and Myf5. EMBO J 22: 5186-5196.
31. Lin YY, Lu JY, Zhang J, Walter W, Dang W, et al. (2009) Protein acetylation microarray reveals that NuA4 controls key metabolic target regulating gluconeogenesis. Cell 136: 1073-1084.

Chapter 5

A NOVEL RNAI LETHALITY RESCUE SCREEN TO IDENTIFY REGULATORS OF ADIPOGENESIS

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ABSTRACT
Adipogenesis, the differentiation of fibroblast-like mesenchymal stem cells into mature adipocytes, is tightly regulated by a complex cascade of transcription factors, including the nuclear receptor Peroxisome proliferator activator receptor γ (PPARγ). RNAi-mediated knock down libraries may present an atractive method for the identification of additional adipogenic factors. However, using *in vitro* adipogenesis model systems for high-throughput screening with siRNA libraries is limited since (i) differentiation is not homogeneous, but results in mixed cell populations, and (ii) the expression levels (and activity) of adipogenic regulators is highly dynamic during differentiation, indicating that the timing of RNAi-mediated knock down during differentiation may be extremely critical. Here we report a proof-of-principle for a novel RNAi screening method to identify regulators of adipogenesis that is based on lethality rescue rather than differentiation, using microRNA expression driven by a PPAR responsive RNA polymerase II promoter. We validated this novel method through screening of a dedicated deubiquitinase knock down library, resulting in the identification of UCHL3 as an essential deubiquitinase in adipogenesis. This system therefore enables the identification of novel genes regulating PPARγ-mediated adipogenesis in a high-throughput setting.

INTRODUCTION

The relationship between obesity and its complications, such as type 2 diabetes and cardiovascular diseases, has firmly established adipose tissue as a key regulator of glucose and lipid metabolism [1]. Adipose tissue regulates metabolism through at least two different mechanisms: the storage of lipids (as triglycerides) and the secretion of so-called adipokines, which function in an endocrine or paracrine fashion. Expansion of adipose tissue, as seen in obese individuals, not only affects the storage of lipids as triglycerides in lipid droplets, but also results in qualitative and quantitative changes in a number of adipokines [2]. The amount of mature adipocytes is largely determined by the differentiation of fibroblast-like mesenchymal stem cells into adipocytes, a process called adipogenesis [1,3]. One of the best-established model systems for adipogenesis is the mouse 3T3-L1 cell line, which can efficiently be differentiated into mature adipocytes by hormonal stimulation under experimental conditions [4,5]. Adipogenesis is regulated by a cascade of transcription factors, ultimately leading to the induction of the transcription factor Peroxisome proliferator activator receptor γ (PPARγ) [1,6]. Several independent lines of investigation have led to the qualification of PPARγ as the master regulator of adipogenesis. For example, *in vitro* differentiation of fibroblasts into mature adipocytes can be induced by introduction of PPARγ [7]. In addition, this protein regulates a large set of "adipocyte genes", involved in lipid and glucose metabolism, in a feed-forward loop with another transcription factor, C/EBPα [8,9]. Furthermore, PPARγ $^{-/-}$ mice are severely lipodystrophic, while PPARγ $^{+/-}$ mice have reduced amounts of adipose tissue [10,11,12,13]. PPARγ is also essential for the maintenance of adipose tissue, since conditional knock-out of the *Pparg* gene resulted in reduced *in vivo* survival of mature adipocytes [14]. Finally, human Familial partial lipodystrophy subtype 3 (FPLD3, MIM 604367) patients, harbouring heterozygous mutations in the *PPARG* gene, are characterized by aberrant fat distribution and metabolic disturbances, including insulin resistance and dyslipidemia [15]. Besides PPARγ other proteins are also essential for adipogenesis, including transcription factors (e.g. KLF5 [16]), transcriptional co-regulators (e.g. TRAP220/Med1 [17], Tip60 [18], TLE3 [19] and TRIP3 [20]) and lipid droplet proteins (e.g. CIDEC/Fsp27 [21]). These essential adipogenic factors have been identified through various means, such as *in vivo* studies [16,22], yeast

2-hybrid screening [17], peptide interaction assays [20], cDNA library high-throughput-screening [19] and co-immunoprecipitations followed by mass spectrometry [18]. Furthermore, phenotypic screening resulted in the identification of the small molecules harmine and phenamil as novel PPARγ-targeting compounds [23,24].

Since the discovery of siRNA as a way to specifically down-regulate gene expression, a broad spectrum of siRNA libraries have been developed that are now widely used in discovering novel protein interactions and to unravel the signalling cascades playing part in various cellular processes [25]. Recent findings indicate that this technology presents an attractive alternative and complementary method for the identification of novel regulators in adipogenesis. Tang *et al.* employed a small 96 well-scale siRNA library screen to identify protein kinases involved in inhibition of insulin induced glucose uptake in fully differentiated 3T3L1 adipocytes. From this screen the integrin-linked protein kinase MAP4K4 was identified as a negative regulator of adipogenesis supressing expression of the adipogenic transcription factors C/EBPα, C/EBPβ and PPARγ [26].

However, while 3T3-L1 cells present one of the best-established models for adipogenesis, screening for novel adipogenic regulators in these cells presents several (potential) problems. First, 3T3-L1 differentiation is not homogeneous, but results in mixed cell populations, with various degrees of differentiation [27,28,29,30,31]. This, together with the genetic variation observed in these cells [32], may result in a high false discovery rate. Second, the expression levels (and activity) of adipogenic regulators is highly dynamic during differentiation [3,6], indicating that the timing of RNAi-mediated knock down during differentiation may be extremely critical. We therefore developed a novel screening method to identify regulators of adipogenesis that makes use of a U2OS cell line stably expressing PPARγ instead of 3T3-L1 cells. This method is primarily based on loss of blasticidin resistance via PPARγ-driven microRNA expression, with the additional advantage that overexpression of PPARγ may inhibit U2OS cell growth, as observed in other cell lines [33]. In conclusion this system uses lethality rescue rather than differentiation as a read-out method, thereby allowing the identification of novel genes regulating adipogenesis in a high-throughput fashion.

RESULTS

PPARγ2 driven miRNA expression results in loss of blasticidin resistance

While vector-based siRNA techniques generally employ RNA polymerase III-driven expression [34], Stegmeier *et al.* recently reported efficient knock-down of gene expression from RNA polymerase II-driven miR30 miRNA-based vectors. [35]. This was achieved by replacing the region encoding the mature miR30 miRNA with sequences that encode shRNAs targeting a gene of choice. Amongst others, this system was tested using two pPRIME (potent RNA interference using microRNA expression) vectors expressing different miRNAs directed against firefly luciferase mRNA, named FF2 and FF3. Based on these vectors, we developed a novel screening method depicted here as "RNAi lethality rescue screening " (Fig. 1).

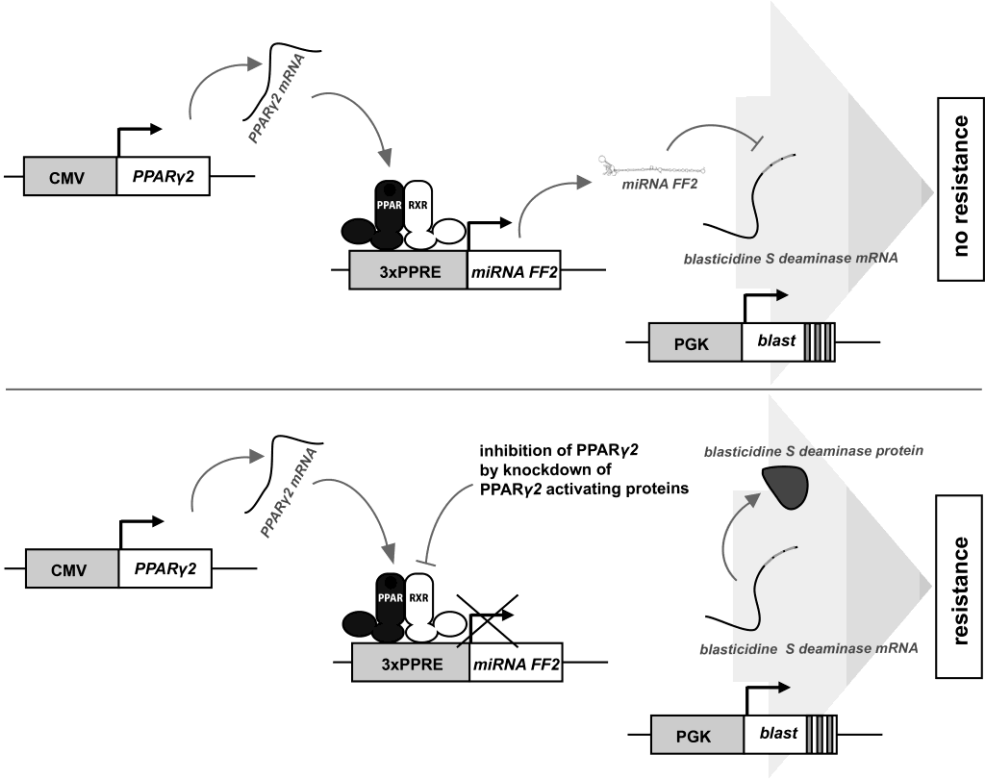

Figure 1. Schematic model of miRNA mediated siRNA screening. The upper panel illustrates the situation of cells with active PPARγ2-mediated gene expression. PPARγ drives miRNA FF2 expression, resulting in repression of the Blasticidin S

deaminase expression cassette via the target repeat FF2 sequences present in the 3' UTR. The lower panel depicts the situation of cells with an siRNA mediated knock-down of a PPARγ2 activating protein. Expression of the FF2 miRNA is diminished and Blasticidin S deaminase expression is no longer repressed, resulting in blasticidin resistance.

For our subsequent studies we used the human U2OS osteosarcoma cell line, since these cells (i) are easy to handle and transfect, (ii) express a robust transcriptional response upon introduction of PPARγ, both on the level of reporter genes [36,37] and endogenous target genes (data not shown), and (iii) may be growth inhibited by activation of PPARγ, a phenomenon also observed in multiple other cell lines [33,38] which will support lethality rescue screening. We first tested whether miRNA FF2, originally directed against *luciferase* mRNA, could target a heterologous transcript. For this, 3 firefly luciferase sequences recognized by miRNA FF2 were fused to the 3' UTR of the blasticidin resistance cassette, which encodes a Blasticidin S deaminase gene (*bsd* from *Aspergillus terreus*). Next, U2OS cells underwent retroviral transduction with a vector encoding this CMV promoter-driven blasticidin-3xFF2 cassette and stable clones were selected with Blasticidin S (Fig. 2A). To test whether constitutive miRNA FF2 expression resulted in loss of resistance, these cells were subsequently transduced with a miRNA FF2 expressing retrovirus, under control of either the RNA polymerase II-driven CMV promoter or the RNA polymerase III-driven U6 promoter. Cells expressing miRNA FF2 driven by either RNA polymerase II or RNA polymerase III showed a significant loss of blasticidin resistance (Fig. 2A). As a control, empty virus or non-specific miRNA FF3 expressing virus were used, and neither resulted in significant loss of blasticidin resistance. From these experiments we conclude that this system allows efficient expression of miRNAs by RNA polymerase II and III promoters. Furthermore, the FF2 targeting sequences, originally from the firefly luciferase gene, can be transferred to a heterologous blasticidin resistance gene, resulting in significant loss of expression of this gene when exposed to the miRNA-based FF2 vector.

PPARγ2 dependent miRNA expression

Next we investigated whether the constitutively active CMV promoter, driving miRNA FF2 expression, could be replaced by a PPARγ2 responsive promoter (see also Fig. 1). We first isolated U2OS cells

stably transduced with a 3xPPRE miRNA FF2 virus. The resulting U2OS 3xPPRE miRNA FF2 stable cell line underwent a second round of retroviral transduction to express Blasticidin S deaminase and PPARγ2 (Fig. 2B). When these two viruses were used at a ratio of 1:10 respectively, activation of PPARγ by rosiglitazone had no effect on the blasticidin resitance. However, when PPARγ expression was increased by shifting the ratio 3xPPRE FF2-Blasticidin: PPARγ2 to 1:25, a significant loss of resistance for Blasticidin S was observed in the presence of 1μM rosiglitazone (Fig. 2C). These experiments indicate that miRNA-FF2 expression from the 3xPPRE-miRNA-FF2 vector can be induced by rosiglitazone in cells expressing sufficient amounts of PPARγ2, resulting in loss of blasticidine resistance conferred by the blasticidine-3xFF2 cassette.

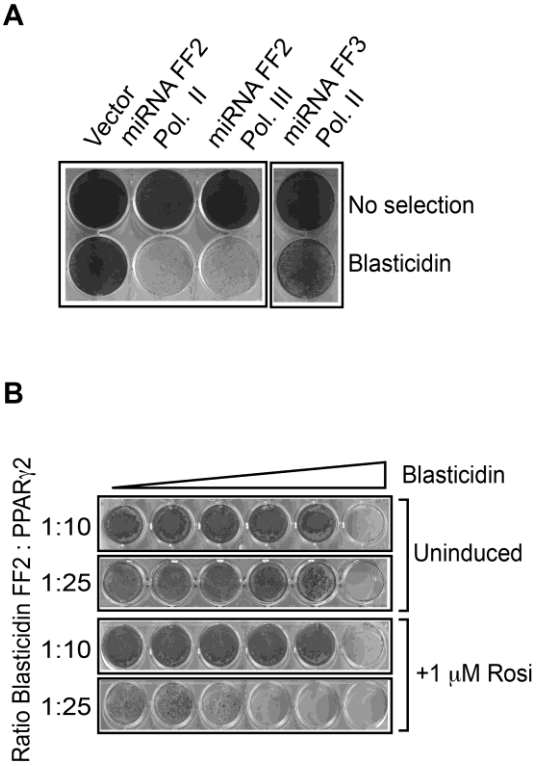

Figure 2. A RNA polymerase II and RNA polymerase III driven miRNA FF2 expression results in loss of Blasticidine resistance. U2OS cells expressing the Blasticidin 3xFF2 expression cassette were transduced with miRNA FF2 expressing virus and selected (50μg/ml Blasticidin S) for 1 week. As a control

either empty virus or not FF2 specific miRNA FF3 virus was used, the latter expressing miRNA not targetting the modified 3'UTR of the blasticidin S deaminase gene. B. PPARγ2 expression compared to empty vector control as detected in the stably transfected U2OS cell line also expressing the blasticidin 3xFF2 expression cassette C. U2OS cells stably transduced with 3xPPRE miRNA FF2 virus were retransduced with blasticidin 3xFF2 virus and PPARγ2 virus at a ration of 1:10 or 1:25 respectively. Cells transduced at a ratio of 1:25 show loss of resistance when incubated in 1μM rosiglitazone and increasing amounts of Blasticidin S.

To verify whether the effect of PPARγ2-driven miRNA expression on loss of blasticidine resistance is indeed mediated by PPARγ2, we knocked-down PPARγ2 expression by siRNA technology. Different shRNA expression vectors directed against PPARγ were generated and transiently expressed in U2OS 3xPPRE miRNA FF2 cells. As shown in Figure 3A (upper panel), siRNA vector #4 efficiently knocked-down the expression of PPARγ while vectors #1-3 were less efficient, as assesed by Western blotting. Next, U2OS 3xPPRE miRNA FF2 cells were transiently transfected with the different PPARγ siRNA expression vectors and incubated in the presence of rosiglitazone. After selection with Blasticidin S for 1 week in the presence of rosiglitazone (1μM), colonies were visualized with Giemsa staining. As shown in Figure 3A (lower panel), the functional PPARγ siRNA expression vector #4 could partially rescue the loss of blasticidine resistance observed upon activation of PPARγ by rosiglitazone. Loss of blasticidin resistance was only partially rescued by considerable knock down of PPARγ activity, suggesting that the screening method filters out mainly strong activators of the PPARγ pathway. The 3 shRNA vectors which gave no efficient knock-down of PPARγ expression (#1, #2 and #3) also failed to rescue blasticidine resistance (Fig. 3A), indicating that the loss of blasticidin resistance in the presence of rosiglitazone shown in Figure 2B is indeed mediated by PPARγ. To corroborate these findings, FACS analysis was performed on the same cells with different concentrations of Blasticidin. For this we developed a retroviral GFP vector expressing the functional siRNA #4 directed against PPARγ2 described above. U2OS 3xPPRE miRNA FF2 cells were stably transduced and selected at different concentrations of Blasticidin S for 1 week. The percentage of GFP positive cells was determined by FACS analysis. The percentage of GFP postive cells after Blasticidin S selection increased approximately three fold in case of siRNA induced PPARγ2 knock down while it

remained unchanged in empty GFP vector transduced cells (Fig. 3B). Taken together, these experiments indicate that the loss of resistance in U2OS 3xPPRE miRNA FF2 induced by rosiglitazone is dependent on PPARγ2 expression.

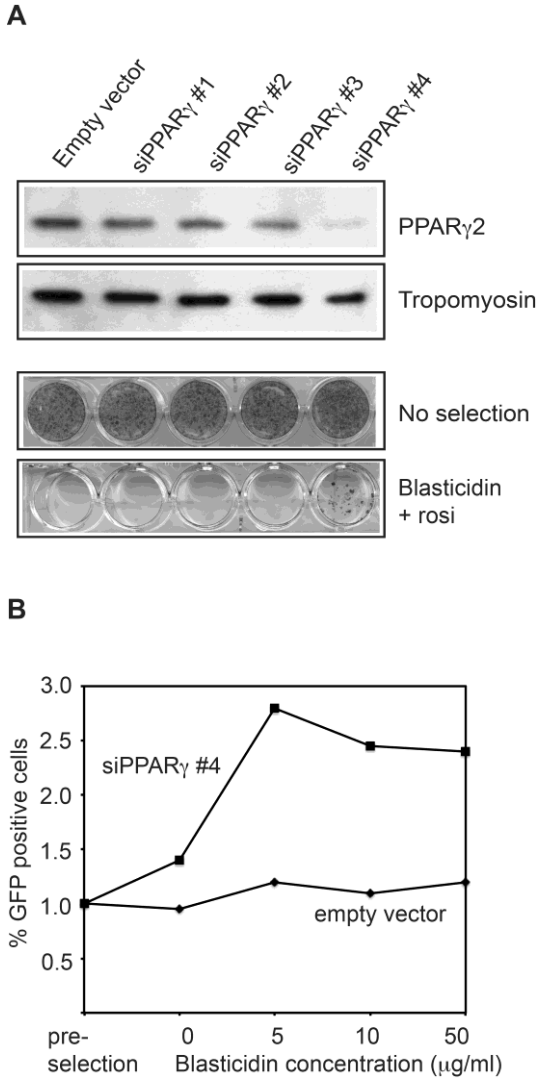

Figure 3. A siRNA-mediated knock down of PPARγ rescues miRNA FF2 mediated loss of blasticidin resistance. Different PPARγ specific siRNA vectors were generated and tested for their ability to rescue the loss of blasticidin resistance. The functional siRNA vector #4 rescues the miRNA FF2 induced loss of resistance. B. The U2OS 3xPPRE miRNA FF2 cells were partially rescued with PPAR#4 siRNA

expressing GFP virus. The percentage of GFP positive cells was determined using FACS analysis after 1 week of blasticidin selection at various concentrations.

RNAi lethality rescue screening identifies UCHL3 as a regulator of adipogenesis

Post-translational modifications (PTM), such as phosphorylation, acetylation, sumoylation and ubiquitination, can regulate the transcriptional output of adipogenic transcription factors like C/EBPα and PPARγ (reviewed in [39,40]). Since the role of deubiquitylating enzymes in adipogenesis has not been studied extensively yet, we used an shRNA library that targets deubiquitinating enzymes (DUBs) [41] to validate our screening system. Four shRNA knockdown vectors against each DUB were pooled into 24 sets, where each set targets a single DUB transcript (see Table S1). The U2OS 3xPPRE miRNA FF2 cells were selected by Blasticidin S in the presence of 1μM rosiglitazone after siRNA vector electroporation. After 3 weeks of culture, colony formation was only observed when the expression of Ubiquitin Carboxyl-terminal Hydrolase isozyme L3 (UCHL3) or UCHL5 was reduced, while for example knock down of the closely related UCHL1 enzyme did not result in colony formation (Fig. 4A).

To further investigate the potential role of UCHL3 in adipogenesis, we first examined its protein expression and localization in 3T3-L1 cells. As is shown in Figure 4B, the expression levels of UCHL3 increased during 3T3-L1 differentiation. Next, we examined the subcellular localisation of UCHL3 in mature 3T3-L1 adipocytes. In agreement with other cell types [42,43,44], UCHL3 displayed cytoplasmatic localization in 3T3-L1 adipocytes (Fig. 4D). Finally, we addressed the relevance of UCHL3 in adipogenesis using two different approaches. First, UCHL3 enzymatic activity was determined, using an HA-tagged ubiquitin probe. This probe is recognized by active DUBs, after which the reactive group attached to the probe covalently and irreversibly binds DUBs [45,46]. Immunoprecipitation of active DUBs from mature 3T3-L1 adipocytes using anti-HA antibodies followed by Western blotting with UCHL3 antibodies showed that UCHL3 is an enzymatically active DUB in mature adipocytes (Figure 4D). To address the relevance of UCHL3 in adipogenesis, the expression of this protein was reduced. Adipogenesis was inhibited by lentiviral short hairpin constructs against UCHL3 (shUCHL3 #14, 15 and 16), as illustrated by triglyceride staining with Oil-red-O (Fig. 4E) and PPARγ and FABP4 expression (Fig. 4F). The

degree of inhibition correlated with the reduction in UCHL3 protein expression, with shUCHL3 #14 being the most efficient and shUCHL3 #15 displaying a partial effect, and underscores the importance of UCHL3 in 3T3-L1 adipocyte differentiation. Taken together, our findings indicate that novel regulators of adipogenesis can be identified by the RNAi lethality rescue screening method described here.

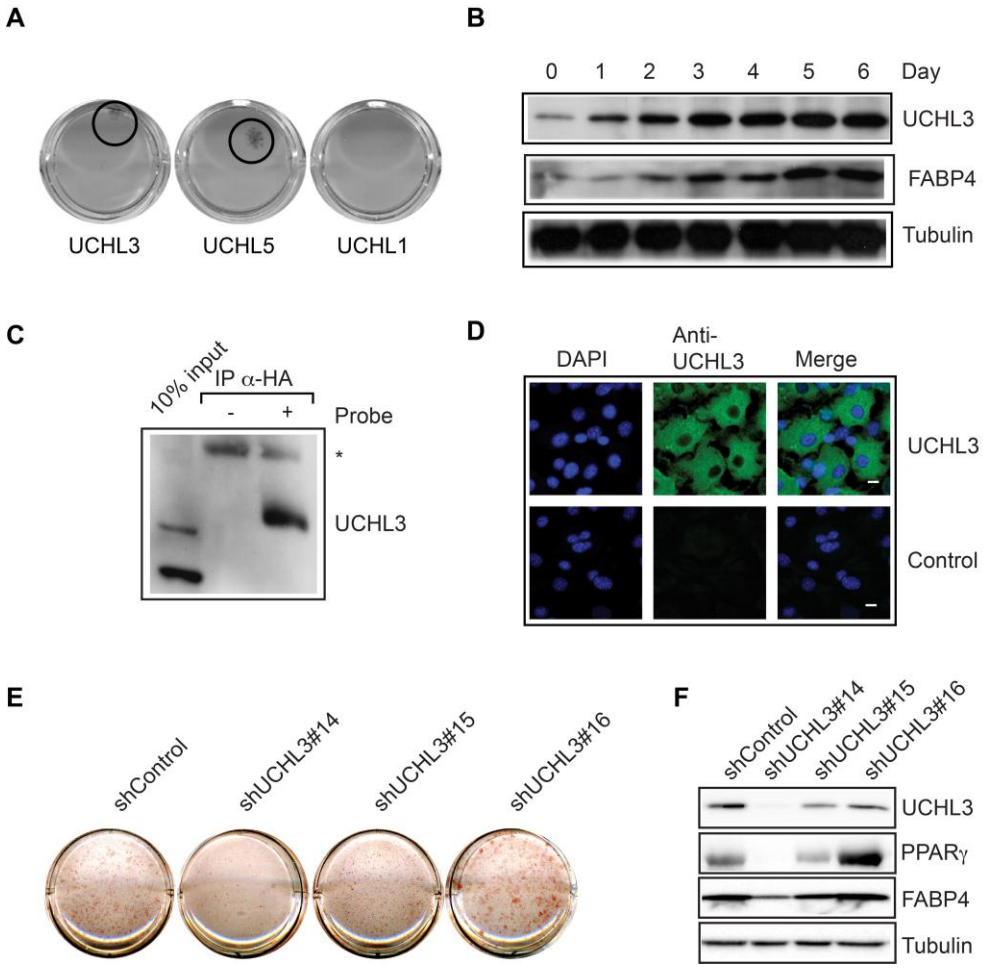

Figure 4. A, Small siRNA DUB screen perfomed in U2OS 3xPPRE miRNA FF2 cells. A partial siRNA library against 24 different deubiquitinating enzymes was tested for the ability to rescue the miRNA FF2 induced loss of Blasticidin resistance. Knock down of UCHL3 and UCHL5, but not UCHL1, resulted in colony formation. B. UCHL3 expression increases during 3T3-L1 adipocyte differentiation. Mouse

3T3L1 preadipocytes were differentiated into mature adipocytes and samples were taken at different time points during differentiation. Protein expression levels of UCHL3 were determined by Western blot analysis. As control for differentiation Fabp4 protein levels were analysed. C. UCHL3 activity in 3T3-L1 adipocytes. Cell lysates of differentiated 3T3-L1 cells (day 6) were incubated with or without HA-Ub probe, DUB activities were immunprecipitated (anti-HA agarose) and UCHL3 was detected by Western blotting. Note the difference in mobility between unmodified UCHL3 (input lane) and UCHL3 covalently bound to the DUB probe. An aspecific band is indicated (*). D. Localization of UCHL3 in differentiated 3T3-L1 cells. Nuclei were stained with DAPI, UCHL3 was visualized by indirect immunofluorescence. Merged pictures demonstrate the predominant cytoplasmic localization of UCHL3. As a control, the primary antibody was ommited. Bar, 10 μm. E. 3T3-L1 cells, stably transduced with lentiviral constructs expressing short hairpin RNAs directed against UCHL3 or control shRNA, were subjected to differentiation conditions. At day 10 of differentiation, cells were fixed and stained for triglycerides using Oil-red-O. Pictures are representative for three independent experiments. F. 3T3-L1 cells were stably transduced with either control or UCHL3 shRNA and differentiated as described under E. Cell lysates were subjected to Western blot analysis, using antibodies against UCHL3, PPARγ, FABP4 and tubulin.

DISCUSSION

To identify novel regulators of adipogenesis, we have developed an RNAi lethality rescue screen. This method is based on cell survival, which is accomplished through inhibition of the adipogenic transcription factor PPARγ, resulting in blasticidin resistance and possibly inhibition of PPARγ-mediated growth arrest [33]. A cell survival-based method has the advantage over, for example reporter based screening methods, that it can potentially be used for screening of pooled RNAi libraries. Validation of the RNAi lethality rescue screen described here enabled us to identify UCHL3 as a regulator of PPARγ-mediated differentiation of 3T3-L1 preadipocytes. In agreement with this, *Uchl3*$^{-/-}$ MEFs were very recently shown to display impaired adipocyte differentiation and lipid accumulation [47]. Morever, *Uchl3*$^{-/-}$ mice displayed a reduction of adipose tissue mass and were protected against high-fat diet-induced obesity and insulin resistance [47,48]. Together with the current study, these findings strongly support a critical role for UCHL3 in adipogenesis, both *in vitro* and *in vivo*. Interestingly, the critical role of UCHL3 in adipogenesis may not be limited to its ubiquitin hydrolase activity, as this protein has dual specificity for ubiquitin and Nedd8, a ubiquitin-like protein *in vitro* [49,50] and *in vivo* [51]. Like other post-translational modifications, neddylation can alter substrate function and activity by inducing conformational changes, or by preventing or inducing protein-protein interactions [52]. The critical deubiquitination and/or deneddylation substrates of UCHL3 in adipogenesis remain to be established. Suzuki *et al.* have shown that UCHL3 enhances insulin signalling in (pre)adipocytes, but UCHL3 is unlikely to target critical components of insulin signalling like the insulin receptor, the IGF-I receptor, IRS-1 and Grb10[47]. Interestingly, these authors also observed impaired expression of the late adipogenic genes *fabp4*, *adiponectin* and *srebp1c*, three direct target genes of PPARγ [8,53,54], in *Uchl3*$^{-/-}$ MEFs and epididymal WAT of *Uchl3*$^{-/-}$ mice [47]. Ectopic expression of UCHL3, but not the catalytic mutant C95S, restored expression of these genes in *Uchl3*$^{-/-}$ MEFs [47]. Together with the screening method presented here, which is based on PPARγ activity, these findings suggest that PPARγ may be a direct target for UCHL3. However, in agreement with other studies [42,43,44], we found UCHL3 to be localized in the cytoplasm, while PPARγ was exclusively nuclear in mature 3T3-L1 adipocytes. In addition, recombinant UCHL3 failed to deubiquitinate PPARγ *in vitro*

(Figure S1 and Materials and Methods SI), indicating that UCHL3 modulates PPARγ activity by an indirect mechanism, either through its ubiquitin hydrolase or its deneddylase activity.
Taken together, we have developed and validated a novel RNAi screen, based on PPARγ induced growth arrest and loss of resistance. This screening method was validated on a small scale using an shRNA library targeting different deubiquitinating enzymes. From these initial screens we identified UCHL3 as a regulator of adipogenesis. In the future PPARγ-mediated RNAi lethality rescue screening may allow high throughput screening of pooled RNAi libraries. The modules of this system can also readily be exchanged for other reporters and/or expression vectors, including heterologous reporters in combination with fusions of protein domains (Gal4, LexA) (Fig. 1). Modified forms of this lethality rescue screening method may therefore present a more generic tool to identify regulatory proteins in fundamental cellular processes.

MATERIALS AND METHODS

Materials

RNAiMax was purchased from Invitrogen (Carslbad, USA). The following antibodies were used: anti-PPARγ (sc-7273), Santa Cruz Biotechnologies; anti-UCHL3 (3525), Cell Signalling Technology; anti-tubulin (ab6046) Abcam; anti-rabbit-HRP (111035144) and anti-mouse-HRP (115035146), Jackson Immunoresearch Laboratories Inc.; mouse anti-rabbit Alexa488, Invitrogen. The vinyl methyl ester HA-DUB probe (HAUbVME) was generated and used as described [45]

Plasmids

The retroviral expression plasmid pMSCV-mPPARγ2 (puro) was a kind gift from Dr. B.M. Spiegelman [55]. The pPrime miRNA expression CMV miRNA FF2 and FF3 vectors were a kind gift from the Elledge lab and used for subsequent cloning [35]. Target sequence with a mismatch at the first base for FF2; cCCGCCTGAAGTCTCTGATTAA and for FF3; aGCTCCCGCTGAATTGGAATCC. A *Bgl*II-*Hind*III fragment encompassing the 3xPPRE-TK promoter was digested from the 3xPPRE-TK-luciferase reporter and subcloned into pLNCX Δ*Cla*I. The miRNA FF2 cassette was digested from pPrime using *Cla*I, *Not*I and subcloned behind the 3xPPRE-TK promoter to generate the pLNCX 3xPPRE FF2miRNA retroviral vector. A SV40 polyA signal oligo was ligated at the 3' end of the FF2 cassette, using a *Cla*I site. All recombinant DNA work was performed according to standard procedures [56]. All mutations were generated by Quickchange mutagenesis (Stratagene) and verified by sequencing.

Cell culture and differentiation assays

The human osteosarcoma cell line U2OS (ATCC, Manassas, VA) and the Phoenix amphotropic packaging cell line (Allel Biotechnology, San Diego, CA) were maintained in DMEM Glutamax (Dulbecco) containing 10% foetal calf serum (Gibco Life Technologies), 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco Life Technologies). Electroporations of U2OS cells were performed with the Biorad Genepulser Xcell using 2 μg plasmid DNA and 100ul cell suspension in electroporation buffer (2mM Hepes pH 7.2, 15mM K_2PO_4/KH_2PO_4, 250mM Manitol and 1mM $MgCl_2$ pH 7.2) per electroporation, with two times 8 pulses at 140V, 1.5 msec burst duration at intervals of 1.5 s.

After 1 week of Blasticidin S selection at different concentrations (0, 5, 10 and 50 μg/ml) cells were either fixed and subjected to Giemsa staining or trypsinized and subsequently used for FACS analysis. After brief centrifugation cells were resuspended in ice cold PBS. The percentage of GFP positive cells of the total cell population was determined after counting 10.000 cells using a FACScan (Becton Dickinson, Biosciences).
Lentivirusses for transduction of UCHL3 short hairpin constructs were generated in HEK293T cells using the Mission® system (Sigma-Aldrich). As control the pLKO.1-puro Non-Mammalian shRNA Control plasmid (SHC002) was used. After lentiviral transduction, shRNA expressing cells were selected by puromycin selection. Differentiation of shRNA expressing 3T3-L1 cells, Oil-red-O staining and Western blotting were performed as described [18,20].

Immunofluorescence
For immunofluorescence staining, 3T3-L1 cells were plated on glass coverslips. Cells were differentiated for 5 days, fixed with 4% paraformaldehyde for 20 min RT and permeabilized in PBS supplemented with 0.5% Triton for 10 min. After 30 min incubation in blocking buffer, cells were stained with primary antibodies for 2h at RT, then incubated with secondary fluorochrome-conjugated antibodies. After several washes, coverslips were mounted in Dabco-DAPI and analyzed with an LSM710 Met confocal microscope (Carl Zeiss, Jena, Germany).

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REFERENCES

1. Rosen ED, Spiegelman BM (2006) Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444: 847-853.
2. Guilherme A, Virbasius JV, Puri V, Czech MP (2008) Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. NatRevMolCell Biol 9: 367-377.
3. Lefterova MI, Lazar MA (2009) New developments in adipogenesis. Trends EndocrinolMetab 20: 107-114.
4. Green H, Kehinde O (1974) Sublines of mouse 3T3 cells that accumulate lipid. Cell 1: 113-116.
5. Poulos SP, Dodson MV, Hausman GJ (2010) Cell line models for differentiation: preadipocytes and adipocytes. ExpBiolMed(Maywood) 235: 1185-1193.
6. Farmer SR (2006) Transcriptional control of adipocyte formation. Cell Metab 4: 263-273.
7. Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79: 1147-1156.
8. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, et al. (2008) Genome-wide profiling of PPAR{gamma}:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev 22: 2953-2967.
9. Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, et al. (2008) PPAR{gamma} and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes Dev 22: 2941-2952.
10. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, et al. (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. MolCell 4: 585-595.
11. Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, et al. (1999) PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. MolCell 4: 597-609.
12. Nadra K, Quignodon L, Sardella C, Joye E, Mucciolo A, et al. (2010) PPARgamma in placental angiogenesis. Endocrinology 151: 4969-4981.
13. Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, et al. (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. MolCell 4: 611-617.

14. Imai T, Takakuwa R, Marchand S, Dentz E, Bornert JM, et al. (2004) Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. ProcNatlAcadSciUSA 101: 4543-4547.
15. Hegele RA (2005) Lessons from human mutations in PPARgamma. IntJObes(Lond) 29 Suppl 1:S31-5.: S31-S35.
16. Oishi Y, Manabe I, Tobe K, Tsushima K, Shindo T, et al. (2005) Kruppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. Cell Metab 1: 27-39.
17. Zhu Y, Qi C, Jain S, Rao MS, Reddy JK (1997) Isolation and characterization of PBP, a protein that interacts with peroxisome proliferator-activated receptor. JBiolChem 272: 25500-25506.
18. van Beekum O, Brenkman AB, Grontved L, Hamers N, van den Broek NJ, et al. (2008) The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARgamma. Endocrinology 149: 1840-1849.
19. Villanueva CJ, Waki H, Godio C, Nielsen R, Chou WL, et al. (2011) TLE3 Is a Dual-Function Transcriptional Coregulator of Adipogenesis. Cell Metab 13: 413-427.
20. Koppen A, Houtman R, Pijnenburg D, Jeninga EH, Ruijtenbeek R, et al. (2009) Nuclear receptor-coregulator interaction profiling identifies TRIP3 as a novel PPARgamma cofactor. MolCell Proteomics 8: 2212-2226.
21. Li F, Gu Y, Dong W, Li H, Zhang L, et al. (2010) Cell death-inducing DFF45-like effector, a lipid droplet-associated protein, might be involved in the differentiation of human adipocytes. FEBS J 277: 4173-4183.
22. Louet JF, Coste A, Amazit L, Tannour-Louet M, Wu RC, et al. (2006) Oncogenic steroid receptor coactivator-3 is a key regulator of the white adipogenic program. ProcNatlAcadSciUSA 103: 17868-17873.
23. Park KW, Waki H, Choi SP, Park KM, Tontonoz P (2010) The small molecule phenamil is a modulator of adipocyte differentiation and PPARgamma expression. JLipid Res 51: 2775-2784.
24. Waki H, Park KW, Mitro N, Pei L, Damoiseaux R, et al. (2007) The small molecule harmine is an antidiabetic cell-type-specific regulator of PPARgamma expression. Cell Metab 5: 357-370.
25. Bernards R, Brummelkamp TR, Beijersbergen RL (2006) shRNA libraries and their use in cancer genetics. NatMethods 3: 701-706.
26. Tang XQ, Guilherme A, Chakladar A, Powelka AM, Konda S, et al. (2006) An RNA interference-based screen identifies MAP4K4/NIK as

a negative regulator of PPAR gamma, adipogenesis, and insulin-responsive hexose transport. ProcNatlAcadSciUSA 103: 2087-2092.
27. Green H, Kehinde O (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5: 19-27.
28. Nagayama M, Uchida T, Gohara K (2007) Temporal and spatial variations of lipid droplets during adipocyte division and differentiation. JLipid Res 48: 9-18.
29. Shigematsu S, Miller SL, Pessin JE (2001) Differentiated 3T3L1 adipocytes are composed of heterogenous cell populations with distinct receptor tyrosine kinase signaling properties. JBiolChem 276: 15292-15297.
30. Loo LH, Lin HJ, Singh DK, Lyons KM, Altschuler SJ, et al. (2009) Heterogeneity in the physiological states and pharmacological responses of differentiating 3T3-L1 preadipocytes. JCell Biol 187: 375-384.
31. Nan X, Cheng JX, Xie XS (2003) Vibrational imaging of lipid droplets in live fibroblast cells with coherent anti-Stokes Raman scattering microscopy. JLipid Res 44: 2202-2208.
32. Green H, Kehinde O (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7: 105-113.
33. Altiok S, Xu M, Spiegelman BM (1997) PPAR gamma induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A. Genes Dev 11: 1987-1998.
34. Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. Science 296: 550-553.
35. Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ (2005) A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. ProcNatlAcadSciUSA 102: 13212-13217.
36. Jeninga EH, van Beekum O, van Dijk AD, Hamers N, Hendriks-Stegeman BI, et al. (2007) Impaired peroxisome proliferator-activated receptor gamma function through mutation of a conserved salt bridge (R425C) in familial partial lipodystrophy. MolEndocrinol 21: 1049-1065.
37. Gijsbers L, Man HY, Kloet SK, de Haan LH, Keijer J, et al. (2011) Stable reporter cell lines for peroxisome proliferator-activated

receptor gamma (PPARgamma)-mediated modulation of gene expression. AnalBiochem.
38. Fajas L, Egler V, Reiter R, Miard S, Lefebvre AM, et al. (2003) PPARgamma controls cell proliferation and apoptosis in an RB-dependent manner. Oncogene 22: 4186-4193.
39. van Beekum O, Fleskens V, Kalkhoven E (2009) Posttranslational modifications of PPAR-gamma: fine-tuning the metabolic master regulator. Obesity(SilverSpring) 17: 213-219.
40. Nerlov C (2008) C/EBPs: recipients of extracellular signals through proteome modulation. CurrOpinCell Biol 20: 180-185.
41. Brummelkamp TR, Nijman SMB, Dirac AMG, Bernards R (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappa B. Nature 424: 797-801.
42. Sowa ME, Bennett EJ, Gygi SP, Harper JW (2009) Defining the human deubiquitinating enzyme interaction landscape. Cell 138: 389-403.
43. Sekiguchi S, Kwon J, Yoshida E, Hamasaki H, Ichinose S, et al. (2006) Localization of ubiquitin C-terminal hydrolase L1 in mouse ova and its function in the plasma membrane to block polyspermy. AmJPathol 169: 1722-1729.
44. Osawa Y, Wang YL, Osaka H, Aoki S, Wada K (2001) Cloning, expression, and mapping of a mouse gene, Uchl4, highly homologous to human and mouse Uchl3. BiochemBiophysResCommun 283: 627-633.
45. Borodovsky A, Ovaa H, Kolli N, Gan-Erdene T, Wilkinson KD, et al. (2002) Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. ChemBiol 9: 1149-1159.
46. Ovaa H, Kessler BM, Rolen U, Galardy PJ, Ploegh HL, et al. (2004) Activity-based ubiquitin-specific protease (USP) profiling of virus-infected and malignant human cells. ProcNatlAcadSciUSA 101: 2253-2258.
47. Suzuki M, Setsuie R, Wada K (2009) Ubiquitin carboxyl-terminal hydrolase l3 promotes insulin signaling and adipogenesis. Endocrinology 150: 5230-5239.
48. Setsuie R, Suzuki M, Kabuta T, Fujita H, Miura S, et al. (2009) Ubiquitin C-terminal hydrolase-L3-knockout mice are resistant to diet-induced obesity and show increased activation of AMP-activated protein kinase in skeletal muscle. FASEB J 23: 4148-4157.

49. Wada H, Kito K, Caskey LS, Yeh ET, Kamitani T (1998) Cleavage of the C-terminus of NEDD8 by UCH-L3. BiochemBiophysResCommun 251: 688-692.
50. Hemelaar J, Borodovsky A, Kessler BM, Reverter D, Cook J, et al. (2004) Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. MolCell Biol 24: 84-95.
51. Kwon J, Wang YL, Setsuie R, Sekiguchi S, Sato Y, et al. (2004) Two closely related ubiquitin C-terminal hydrolase isozymes function as reciprocal modulators of germ cell apoptosis in cryptorchid testis. AmJPathol 165: 1367-1374.
52. Rabut G, Peter M (2008) Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep 9: 969-976.
53. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM (1994) mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 8: 1224-1234.
54. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, et al. (2003) Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes 52: 1655-1663.
55. Ge K, Guermah M, Yuan CX, Ito M, Wallberg AE, et al. (2002) Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis. Nature 417: 563-567.
56. Ausubel FM, Brent R, Kingston R, Moore D, Seidman JJ, et al. (1993) Current Protocols in Molecular Biology. New York: John Wiley & Sons.

SUPPORTING INFORMATION LEGENDS

Table S1 Deubiquitinases tested in screening procedure

Figure S1 UCHL3 fails to deubiquitinate PPAR *in vitro*. HEK293T cells were transfected with HA-tagged PPAR expression construct together with histidine-tagged ubiquitin (His-ubi) expression construct and treated with MG132 (3 M). Ubiquitinated proteins were isolated by Ni-NTA precipitation, eluted from the Ni-NTA beads with imidazole and incubated with recombinant UCHL3 enzyme. Ubiquitinated PPAR was detected by Western blotting (anti-HA antibody).

Materials and Methods SI

Supplemental Table I Deubiquitnases tested in screening procedure

	HGNC name	Alternative name	Ensembl gene ID	Gene ID
1	USP12*		ENSG00000152484	219333
2	USP11		ENSG00000102226	8237
3	USP10		ENSG00000103194	9100
4	USP7		ENSG00000171616	7874
5	USP8		ENSG00000138592	9101
6	USP40		ENSG00000085982	55230
7	PAN2	USP52	ENSG00000135473	9924
8	USP46		ENSG00000109189	64854
9	USP31		ENSG00000103404	57478
10	USP48		ENSG00000090686	84196
11	USP30		ENSG00000135093	84749
12	UCHL5#		ENSG00000116750	51377
13	UCHL3		ENSG00000118939	7347
14	UCHL1		ENSG00000154277	7345
15	USP38		ENSG00000170185	84640
16	USP44		ENSG00000136014	84101
17	USP39		ENSG00000168883	10713
18	USP54		ENSG00000166348	159195
19	USP36		ENSG00000055483	57602
20	USP42		ENSG00000106346	76800
21	USP18		ENSG00000099996	24110
22	USP20		ENSG00000136878	10868
23	USP24		ENSG00000162402	23358
24	USP37		ENSG00000135913	57695
	* Ubiquitin specific protease			
	#UBIQUITIN CARBOXYL-TERMINAL HYDROLASE			

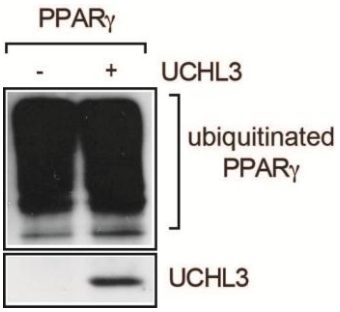

Figure S1 UCHL3 fails to deubiquitinate PPAR *in vitro*. HEK293T cells were transfected with HA-tagged PPAR expression construct together with histidine-tagged ubiquitin (His-ubi) expression construct and M). Ubiquitinated proteins were isolated by Ni-NTA treated with MG132 (3 precipitation, eluted from the Ni-NTA beads with imidazole and incubated with recombinant UCHL3 enzyme. Ubiquitinated PPAR was detected by Western blotting (anti-HA antibody).

Supplemental Materials and Methods

Deubiquitination assays

For *in vitro* (de) ubiquitination assays, cells were transfected with HA-PPAR and His-ubi expression plasmids. Twenty-four hours after transfection, cells were incubated o/n with MG132 (3 M), lysed and his-tagged proteins were isolated as described [1,2]. Recombinant human UCHL3 (E-325; Boston Biochem, Boston, MA) was added to the substrates for 1 hour at 37ºC in deubiquitination buffer (50 mM Tris PH 7.4, 150 mM NaCl, 10 mM DTT, 5 mM $MgCl_2$). Reactions were stopped by adding 2xSDS sample buffer and analysed by Western blotting.

References

1. Stad R, Little NA, Xirodimas DP, Frenk R, Van Der Eb AJ, et al. (2001) Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. EMBO Rep 2: 1029-1034.
2. Meulmeester E, Maurice MM, Boutell C, Teunisse AF, Ovaa H, et al. (2005) Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. MolCell 18: 565-576.

Chapter 6

GENERAL DISCUSSION

Adipocyte differentiation is regulated by a complex network of transcription factors, the activity and expression of which is regulated-amongst others-by transcriptional cofactors, signaling cascades and miRNAs (see Chapter 1). In this thesis we have focused on the transcriptional cofactor and acetyltransferase Tip60, which is essential for *in vitro* differentiation of 3T3-L1 preadipocytes [1] and human primary preadipocytes [2]. Our previous studies had qualified Tip60 as a cofactor for PPARγ, the master regulator of adipogenesis [1]. An interesting observation made at that time was that expression of the Tip60 protein, but not mRNA, increases during the first stages of 3T3-L1 differentiation, suggesting that regulation of Tip60 protein levels may play an important role in early adipogenesis. We therefore wished to investigate the molecular mechanisms regulating Tip60 protein stability in adipogenesis. We identified the deubiquitinase USP7 as an important positive regulator of Tip60 protein levels (Chapter 2). So far, we have not been able to identify the proteins that ubiquitinate Tip60; our findings indicate that the E3 ubiquitin ligase mdm2, which has been implicated in Tip60 degradation in other cellular systems [3], is probably not playing a similar role in preadipocytes (Chapter 3). In addition, we examined the effect heterozygous inactivation of Tip60 *in vivo*. Tip60+/- mice were metabolically healthy, which may be explained by the unaltered Tip60 protein levels in adipose tissue, suggesting compensation by the intact allele (Chapter 4). Finally, we developed a novel RNAi lethality rescue screen to identify regulators of adipogenesis (Chapter 5). As a proof-of principle we screened a small set of deubiquitnases and identified UCHL3 as an essential deubiquitinase in adipogenesis. Together, these findings shed new light on the role of Tip60 in cellular differentiation and provide new insights into the importance of protein (de)ubiquitination in adipogenesis.

Tip60 in cellular differentiation

Role of Tip60 in adipogenesis

Differentiation of 3T3-L1 cells is used as a model system for adipogenesis in our studies. As introduced in Chapter 1, 3T3-L1 differentiation can be divided into 3 parts. First, cells are grown to confluence. Subsequently, cells are exposed to a hormonal cocktail (IBMX, glucocorticoids and insulin) upon which they divide twice in 2 days. This step is called mitotic clonal expansion (MCE). Finally, cells go

into terminal differentiation and start to have the characteristic adipocyte morphology with lipid droplets in the cytoplasm.
Previously, we have implicated Tip60 as an important player in terminal adipocyte differentiation, acting as a coactivator of PPARγ [1]. Importantly, Tip60 recruitment to the PPARγ target genes Fabp4 and perilipin could be observed [1]. Our Tip60 knock down experiments followed by microarray analysis confirm a role for Tip60 in lipid metabolism (Chapter 2). Interestingly, a subset of Tip60 target genes which were also controlled by USP7 were involved in cell cycle regulation, and follow-up experiments support a role for Tip60 in MCE (Chapter 2). This indicates a dual role for Tip60 in adipogenesis: the protein plays a key role in early differentiation (MCE), as well as in the later stages of differentiation. As Tip60 antibodies are suitable for ChIP experiments [1,4] (Chapter 2), it will be interesting to perform genome wide ChIPseq experiments with Tip60 at early and late time points during 3T3-L1 differentiation. While the Tip60 binding profile may display overlap with PPARγ in terminally differentiated cells [5,6], the transcription factor(s) that recruit Tip60 to the DNA in early differentiation are currently unknown. It should be noted that the NuA4/Tip60 complex may also be recruited to target genes through alternative mechanisms, such as H3 lysine 4 trimethylation [7], histone acetylation [8] and RNA polymerase II phosphorylation [4]. Since Tip60 can acetylate histones and non-histone proteins [9], it is tempting to speculate that it is performing its function in adipogenesis at least partly through epigenetic regulation. The critical Tip60 substrates in adipogenesis however remain to be established.

Tip60-USP7 in adipogenesis

Tip60 protein levels are high in preadipocytes and decrease during the pre-differentiation phase [1]. When cells get into MCE, Tip60 protein levels are stabilized by USP7 activity (Chapter 2). Cell cycle exit and differentiation are tightly regulated and coordinated processes [10]. Adipogenesis provides an example where, in order to differentiate quiescent cells first have to go into proliferation again. Whether proliferation is a prerequisite for differentiation and what the link is between differentiation, proliferation and quiescence is of fundamental importance for biological studies. Our findings suggest that Tip60 could have a fundamental role in cell proliferation, since its protein level decreases when cells stop proliferation and increases when cells restart

cycling. USP7 activity and Tip60 could therefore be part of the driving force for cells to re-enter proliferation after cellular quiescence and before adipocyte differentiation. A significant portion of the Tip60-USP7 commonly regulated genes display similar cycling expression patterns during adipogenesis: high expression in preconfluent cells (day -2), a drop in expression upon reaching confluency (day 0), followed by a rise in expression around day 2, after which expression drops again (Chapter 2). These expression profiles correspond to the cell cycle status and also correspond to the Tip60 protein level before and during MCE. However, after MCE, USP7-Tip60 levels stay high while the expression of their common target genes drops (Chapter 2). This suggests that another force suppresses the expression of these genes, drives cell cycle exit and pushes the cells towards terminal differentiation after MCE. One of the candidate proteins to fulfill this role is PPARγ.

Is USP7-Tip60 interplay observed in other cellular processes?

Although there no direct link between USP7 and Tip60 was established previous to our studies, USP7 and Tip60 have since long been indirectly linked with common targets and interactors. Some of the common targets are well known factors in cancer research, which again indicates the importance of these proteins in cell cycle regulation. Of particular interst are the p53, mdm2 an DNMT1 proteins.

USP7 interacts with and stabilizes p53 even in the presence of its E3 ligase Mdm2 [11]. This suggests a tumor suppressor activity for USP7 through the regulation of p53 levels. However, disruption of USP7 also leads to stabilization of p53 [12], which occur through USP7-mediated stabilization of mdm2, an important E3 ubiquitin ligase for p53. This suggests a dynamic role of USP7 in regulation of p53, in which alterations in USP7 levels and/or activity can lead to both increased and decreased p53 protein levels. Interestingly, Tip60 can acetylate p53, which is important for the apoptotic pathway [13], and mdm2 can interact with and ubiquitinate Tip60 [3]. Taken together, a complex interplay between mdm2, USP7 and Tip60 tightly regulates the expression and activity of the tumour suppressor protein p53.

Another protein that is linked to both Tip60 and USP7 is DNA (cytosine-5-)-methyltransferase 1 (DNMT1). This protein has a role in the establishment and regulation of tissue-specific patterns of methylated cytosine residues, which maintains DNA methylation patterns after

cellular replication and sustains proliferative capability in progenitor cells [14]. Tip60 is the acetylase for DNMT1 and the acetylation of DNMT1 leads to the ubiquitination and degradation of DNMT1. USP7 cooperates with HDAC1 and deubiquitinates and stabilizes DNMT1[15]. During DNA replication, the methylation pattern of the parent strand is maintained on the newly synthesized strand through the action of the DNA methyltransferase DNMT1 and DNMT1 is essential for DNA replication and cell proliferation [14]. On the other hand, p53 is a well-known tumor suppressor that stops inappropriate cell cycling. The fact that Tip60 and USP7 are both involved in the regulation of DNMT1 and p53 supports the view that these two enzymes play central roles in fundamental cellular processes like proliferation and differentiation. At the same time, these findings indicate that the interplay between Tip60 and USP7 differs for each cellular setting and process. Whether cell-specific actions and interplay between Tip60 and USP7 are for example associated with differences in posttranslational modifications of the two enzymes needs further investigation [16,17,18,19].

Protein ubiquitination and deubiquitination in adipogenesis

A level of regulation in adipogenesis that has been largely overlooked is protein ubiquitination and deubiquitination. The ubiquitin-proteasome pathway is an important mechanism to regulate protein stability, in which proteins are first ubiquitinated and then degraded by the 26S proteasome. Ubiquitination of substrate proteins involves a cascade of enzymatic reactions. First, ubiquitin is activated by an ubiquitin-activating enzyme (E1), leading to a formation of a thiol-ester bond between the C-terminus of ubiquitin and the E1 enzyme. The activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2). E2 mediates the transfer of ubiquitin to the target protein directly or through an E3-ubiquitin ligase, which transfers ubiquitin to its substrate by covalent attachment between the ubiquitin and lysines on the target protein. E3 ligases thereby determine the substrate specificity of poly ubiquitination. A poly-ubiquitinated chain marks a substrate protein for recognition and eventually degradation by the 26S proteasome [20]. There are multiple families of E3 ligases, such as the RING finger ligases, the HECT domain ligases, and the U box proteins [21,22,23]. A general decline of proteasome activity is observed in adipogenesis in 3T3-L1 cellular models [24]. This is consistent with the observation that during the early stages of differentiation in human

adipose-derived stem cells proteasome activity has been shown to be highest and it decreases as the stem cells become differentiated [25]. At present only a limited number of E3 ubiquitin ligases and substrate proteins have been identified in adipogenesis. Involvement of the ubiquitin-proteasome pathway in adipogenesis can be either negative, through degradation of pro-adipogenic players, or positive, through degradation of anti-adipogenic factors. Pro-adipogenic players that can be targeted by the ubiquitin-proteasome pathway in adipogenesis include C/EBPβ, PPARγ, C/EBPα and Tip60. Overexpression of TRB2, a non-enzymatic signaling intermediate that is down-regulated in early adipogenesis, inhibits adipogenesis by reducing the level of C/EBPβ through a proteasome-dependent way, but the E3 ubiquitin ligase has not been identified [26]. C/EBPα is targeted for degradation by the E3 ligase F-box family member F-box– and WD repeat domain–containing 7 (Fbxw7)[27]. Together with ring-box 1 (Rbx1), cullin 1 (Cul1) and S-phase kinase-associated protein 1 (Skp1), F-box proteins like Fbxw7 form SCF type E3 ubiquitin ligase complexes [23]. Fbxw7-mediated degradation of C/EBPα inhibits adipogenesis [27]. Interestingly, Fbxw7 has also been implicated in lipid metabolism and cell fate decision in mouse liver (Onoyama et al. 342-54). Finally, PPARγ has been identified as a protein that is ubiquitinated and degraded by the proteasome [28]. Very recently, the E3 ubiquitin ligase mSiah1, which also ubiquitnates NCoR1 (see below), was shown to ubiquitnate PPARγ [29]. In Chapter 2 and 3 we show that Tip60 is ubiquitinated and degraded by the ubiquitin-proteasome pathway. While the E3 ligase mdm2 has been shown to ubiquitnate Tip60[3], another enzyme probably ubiquitinates Tip60 in adipogenesis (Chapter 3).

Anti-adipogenic players that can be targeted by the ubiquitin-proteasome pathway include KLF2, NCoR1, Rev-erbAα. KLF2 plays an negative role in adipogenesis by directly inhibiting PPARγ expression [30]. A HECT-domain E3-ubiquitin ligase, WWP1, interacts with KLF2 in vivo and mediates both poly-ubiquitination and proteasomal degradation of KLF2. Thus WWP1 has a positive role in regulation of adipogenesis [31]. The E3 ubiquitin ligase mSiah1 targets NCoR1, a corepressor for PPARγ and other transcription factors [32], for proteasomal degradation[33]. Through regulating the interaction of TRα (thyroid hormone receptor α) and NCoR1, which has an inhibiting effect on the promoter of C/EBPα, mSiah1 plays a positive role in adipogenesis. The third anti-adipogenic factor, which is in fact also pro-

adipogenic, that is targeted by the proteasome is Rev-erbAα, an orphan nuclear receptor [34,35]. The Rev-erbAα protein is necessary for the early mitotic events that are required for adipogenesis. The subsequent reduction in Rev-erbAα protein, due to increased degradation via the 26S proteasome, is also required for adipocyte differentiation because Rev-erbAα represses the expression of PPARγ2, the master transcriptional regulator of adipogenesis [36].

Protein deubiquitination in adipogenesis is largely under-investigated. In Chapters 2 en 5 we investigated deubiquitinase activity during adipogenesis and discovered two major enzymatic activities, corresponding to the cytoplasmatic UCHL3 enzyme and the nuclear USP7 protein. While the cytoplasmatic deubiquitinase UCHL3 has been implicated in adipogenesis and insulin signalling both *in vitro* and *in vivo* [37,38,39], its substrate(s) are currently unknown. In Chapter 2 we describe Tip60 as a USP7 substrate. As the human genome contains only 63 deubiquitinases [40], it seem likely that USP7 substrates other than Tip60 exist in adipocytes.

Taken together, these findings indicate that protein ubiquitination and deubiquitination play important roles in adipogenesis, but many of the critical enzymes and substrates probably remain to be discovered.

References

1. van Beekum O, Brenkman AB, Grontved L, Hamers N, van den Broek NJ, et al. (2008) The adipogenic acetyltransferase Tip60 targets activation function 1 of PPARgamma. Endocrinology 149: 1840-1849.
2. Gronniger E, Wessel S, Kuhn SC, Sohle J, Wenck H, et al. (2010) A new protocol for functional analysis of adipogenesis using reverse transfection technology and time-lapse video microscopy. Cell BiolInt 34: 737-746.
3. Legube G, Linares LK, Lemercier C, Scheffner M, Khochbin S, et al. (2002) Tip60 is targeted to proteasome-mediated degradation by Mdm2 and accumulates after UV irradiation. EMBO J 21: 1704-1712.
4. Wang Z, Zang C, Cui K, Schones DE, Barski A, et al. (2009) Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell 138: 1019-1031.
5. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, et al. (2008) Genome-wide profiling of PPAR{gamma}:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. Genes Dev 22: 2953-2967.
6. Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, et al. (2008) PPAR{gamma} and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes Dev 22: 2941-2952.
7. Fazzio TG, Huff JT, Panning B (2008) An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. Cell 134: 162-174.
8. Shi X, Hong T, Walter KL, Ewalt M, Michishita E, et al. (2006) ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature 442: 96-99.
9. Sapountzi V, Logan IR, Robson CN (2006) Cellular functions of TIP60. IntJBiochemCell Biol 38: 1496-1509.
10. Zhu L, Skoultchi AI (2001) Coordinating cell proliferation and differentiation. Current opinion in genetics & development 11: 91-97.
11. Li M, Brooks CL, Kon N, Gu W (2004) A dynamic role of HAUSP in the p53-Mdm2 pathway. Molecular cell 13: 879-886.
12. Cummins JM, Vogelstein B (2004) HAUSP is required for p53 destabilization. Cell Cycle 3: 689-692.

13. Tang Y, Luo J, Zhang W, Gu W (2006) Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. MolCell 24: 827-839.
14. Qin W, Leonhardt H, Pichler G (2011) Regulation of DNA methyltransferase 1 by interactions and modifications. Nucleus 2: 392-402.
15. Du Z, Song J, Wang Y, Zhao Y, Guda K, et al. (2010) DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. SciSignal 3: ra80.
16. Cheng Z, Ke Y, Ding X, Wang F, Wang H, et al. (2008) Functional characterization of TIP60 sumoylation in UV-irradiated DNA damage response. Oncogene 27: 931-941.
17. Lee HJ, Kim MS, Kim YK, Oh YK, Baek KH (2005) HAUSP, a deubiquitinating enzyme for p53, is polyubiquitinated, polyneddylated, and dimerized. FEBS Lett 579: 4867-4872.
18. Lemercier C, Legube G, Caron C, Louwagie M, Garin J, et al. (2003) Tip60 acetyltransferase activity is controlled by phosphorylation. JBiolChem 278: 4713-4718.
19. Fernandez-Montalvan A, Bouwmeester T, Joberty G, Mader R, Mahnke M, et al. (2007) Biochemical characterization of USP7 reveals post-translational modification sites and structural requirements for substrate processing and subcellular localization. FEBS J 274: 4256-4270.
20. Hershko A, Ciechanover A (1998) The ubiquitin system. Annual review of biochemistry 67: 425-479.
21. Hatakeyama S, Nakayama KI (2003) U-box proteins as a new family of ubiquitin ligases. Biochemical and biophysical research communications 302: 635-645.
22. Pickart CM (2001) Mechanisms underlying ubiquitination. Annual review of biochemistry 70: 503-533.
23. Hindley CJ, McDowell GS, Wise H, Philpott A (2011) Regulation of cell fate determination by Skp1-Cullin1-F-box (SCF) E3 ubiquitin ligases. The International journal of developmental biology 55: 249-260.
24. Dasuri K, Zhang L, Ebenezer P, Fernandez-Kim SO, Bruce-Keller AJ, et al. (2011) Proteasome alterations during adipose differentiation and aging: links to impaired adipocyte differentiation and development of oxidative stress. Free radical biology & medicine 51: 1727-1735.

25. Sakamoto K, Sato Y, Sei M, Ewis AA, Nakahori Y (2010) Proteasome activity correlates with male BMI and contributes to the differentiation of adipocyte in hADSC. Endocrine 37: 274-279.
26. Naiki T, Saijou E, Miyaoka Y, Sekine K, Miyajima A (2007) TRB2, a mouse Tribbles ortholog, suppresses adipocyte differentiation by inhibiting AKT and C/EBPbeta. The Journal of biological chemistry 282: 24075-24082.
27. Bengoechea-Alonso MT, Ericsson J (2010) The ubiquitin ligase Fbxw7 controls adipocyte differentiation by targeting C/EBPalpha for degradation. Proceedings of the National Academy of Sciences of the United States of America 107: 11817-11822.
28. Hauser S, Adelmant G, Sarraf P, Wright HM, Mueller E, et al. (2000) Degradation of the peroxisome proliferator-activated receptor gamma is linked to ligand-dependent activation. JBiolChem 275: 18527-18533.
29. Kilroy G, Kirk-Ballard H, Carter LE, Floyd ZE (2012) The ubiquitin ligase Siah2 regulates PPARgamma activity in adipocytes. Endocrinology 153: 1206-1218.
30. Banerjee SS, Feinberg MW, Watanabe M, Gray S, Haspel RL, et al. (2003) The Kruppel-like factor KLF2 inhibits peroxisome proliferator-activated receptor-gamma expression and adipogenesis. The Journal of biological chemistry 278: 2581-2584.
31. Zhang X, Srinivasan SV, Lingrel JB (2004) WWP1-dependent ubiquitination and degradation of the lung Kruppel-like factor, KLF2. Biochemical and biophysical research communications 316: 139-148.
32. Yu C, Markan K, Temple KA, Deplewski D, Brady MJ, et al. (2005) The nuclear receptor corepressors NCoR and SMRT decrease peroxisome proliferator-activated receptor gamma transcriptional activity and repress 3T3-L1 adipogenesis. JBiolChem 280: 13600-13605.
33. Zhu XG, Kim DW, Goodson ML, Privalsky ML, Cheng SY (2011) NCoR1 regulates thyroid hormone receptor isoform-dependent adipogenesis. Journal of molecular endocrinology 46: 233-244.
34. Chawla A, Lazar MA (1993) Induction of Rev-ErbA alpha, an orphan receptor encoded on the opposite strand of the alpha-thyroid hormone receptor gene, during adipocyte differentiation. The Journal of biological chemistry 268: 16265-16269.

35. Fontaine C, Dubois G, Duguay Y, Helledie T, Vu-Dac N, et al. (2003) The orphan nuclear receptor Rev-Erbalpha is a peroxisome proliferator-activated receptor (PPAR) gamma target gene and promotes PPARgamma-induced adipocyte differentiation. The Journal of biological chemistry 278: 37672-37680.
36. Wang J, Lazar MA (2008) Bifunctional role of Rev-erbalpha in adipocyte differentiation. Molecular and cellular biology 28: 2213-2220.
37. Setsuie R, Suzuki M, Kabuta T, Fujita H, Miura S, et al. (2009) Ubiquitin C-terminal hydrolase-L3-knockout mice are resistant to diet-induced obesity and show increased activation of AMP-activated protein kinase in skeletal muscle. FASEB J 23: 4148-4157.
38. Suzuki M, Setsuie R, Wada K (2009) Ubiquitin carboxyl-terminal hydrolase l3 promotes insulin signaling and adipogenesis. Endocrinology 150: 5230-5239.
39. van Beekum O, Gao Y, Berger R, Koppen A, Kalkhoven E (2012) A Novel RNAi Lethality Rescue Screen to Identify Regulators of Adipogenesis. PloS one 7: e37680.
40. Nijman SMB, Luna-Vargas MPA, Velds A, Brummelkamp TR, Dirac AMG, et al. (2005) A genomic and functional inventory of deubiquitinating enzymes. Cell 123: 773-786.

SUMMARY

Besides playing an important role in providing insulation and protection against mechanical stress, white adipose tissue (WAT) has long been recognized as a storage depot for excess energy. Energy is stored as lipids, which can be hydrolysed in time of caloric need to generate energy. Recently, WAT has been recognized as an endocrine organ, releasing a wide range of adipokines, which for example regulate immune responses, blood pressure control, angiogenesis, haemostasis, bone mass and thyroid and reproductive function. Expansion of white adipose tissue, as seen during the development of increased bodyweight and obesity, involves an increase in adipocyte size and the formation of new adipocytes from precursor cells (adipogenesis). Since adipose tissue is increasingly being recognized as a key regulator of whole-body energy homeostasis and consequently as a prime therapeutic target for metabolic syndrome, adipocyte differentiation and biology are under intensive study. Differentiation of 3T3-L1 fibroblasts is widely used as a model system for adipogenesis and can be divided into three phases. First, preadipocytes are cultured in normal medium till reaching confluence. Second, the confluent adipocytes are cultured for 2-3 days in the presence of a hormonal cocktail, a period referred to as mitotic clonal expansion (MCE) because the preadipocytes re-enter the cell cycle and undergo another two rounds of cell division. Third, cells undergo terminal differentiation to become mature white adipocytes when cultured in the presence of media containing only insulin for another 3-10 days. A complex cascade of transcriptional events regulates adipogenesis, ultimately leading to induction of PPARγ, the key regulator of adipogenesis. Transcription factors like PPARγ require so-called coregulator proteins to regulate gene expression. Recently, we have identified the coregulator protein Tip60 as an essential player in adipogenesis. In this thesis we investigated the exact role of the Tip60 in adipogenesis.

In **Chapter 2** we investigated posttranslational modification of the coregulator protein Tip60 in adipogenesis. Previous studies had indicated that Tip60 is essential for adipogenesis, and that Tip60 protein expression but not mRNA expression was upregulated during adipocyte differentiation. As this suggested to us that the Tip60 protein may be stabilized during adipogenesis, we examined whether the protein is normally degraded by the ubiquitin-proteasome system (UPS)

and protected from degradation by deubiquitination during adipogenesis. We indeed found that expression of the Tip60 protein is regulated through polyubiquitination on multiple residues. USP7, a dominant deubiquitinating enzyme (DUB) in 3T3-L1 adipocytes and mouse adipose tissue, deubiquitinated Tip60 both in intact cells and *in vitro*, and increased Tip60 protein levels. Inhibition of USP7 expression and activity decreased adipogenesis. Transcriptome analysis revealed several cell cycle genes to be co-regulated by both Tip60 and USP7. Knock down of either factor resulted in impaired mitotic clonal expansion, an early step in adipogenesis. These results therefore reveal deubiquitination of a transcriptional coregulator to be a key mechanism in the regulation of early adipogenesis.

Based on the studies in Chapter 2 we decided to try and identify how Tip60 is ubiquitinated in adipocytes in **Chapter 3**. An important step in protein ubiquitination is performed by the so-called E3 ligases, which attach ubiquitin to the target protein and provide specificity to the modification system. The E3 ubiquitin ligase murine double minute 2 (mdm2), an enzyme best known for its major role in regulating tumour suppressor p53 destruction, has been shown to ubiquitinate Tip60 in other cellular contexts, and is highly expressed in (pre)adipocytes. We therefore investigated whether mdm2 is responsible for Tip60 degradation in adipogenesis. In contrast to Tip60 protein, expression of the mdm2 protein, which displayed nuclear localization, as well as mRNA expression were relatively constant during 3T3-L1 adipogenesis. In mdm2-negative MEFs, Tip60 could still be ubiquitinated. In contrast, ubiquitination of p53 was completely dependent on re-introduction of mdm2 in these cells. Together, these findings indicate that E3 ubiquitin ligases other than mdm2 are probably responsible for Tip60 degradation during early adipogenesis.

In **Chapter 4** we investigated the role of Tip60 on adipocyte differentiation and function, and possible consequences on energy homeostasis, *in vivo*. As homozygous inactivation results in early embryonic lethality, Tip60+/- mice were used. Heterozygous inactivation of Tip60 had no effect on body weight, despite higher food intake by Tip60+/- mice. No major effects of heterozygous inactivation of Tip60 were observed on adipose tissue and liver, and Tip60+/- displayed normal glucose tolerance, both on a low fat and a high fat diet. While Tip60 mRNA was reduced to 50% in adipose tissue, the protein levels were unaltered, suggesting compensation by the intact allele.

These findings indicate that the *in vivo* role of Tip60 in adipocyte differentiation and function cannot be properly addressed in Tip60+/- mice, but requires the generation of adipose tissue-specific knock out animals or specific knock-in mice.

In **Chapter 5** we describe the development of a novel method to identify adipogenic factors using RNAi-mediated knock down libraries (siRNA libraries). So far, using in vitro adipogenesis model systems for high-throughput screening with siRNA libraries is limited since (i) differentiation is not homogeneous, but results in mixed cell populations, and (ii) the expression levels (and activity) of adipogenic regulators is highly dynamic during differentiation, indicating that the timing of RNAi-mediated knock down during differentiation may be extremely critical. In Chapter 5 we report a proof-of-principle for a novel RNAi screening method to identify regulators of adipogenesis that is based on lethality rescue rather than differentiation, using microRNA expression driven by a PPARγ responsive RNA polymerase II promoter. We validated this novel method through screening of a dedicated deubiquitinase knock down library, resulting in the identification of UCHL3 as an essential deubiquitinase in adipogenesis. This system therefore enables the identification of novel genes regulating PPARγ-mediated adipogenesis in a high-throughput setting.

SAMENVATTING

Vetweefsel speelt een belangrijke rol in de isolatie van ons lichaam, als bescherming tegen mechanische stress en als opslagorgaan voor energie. Energie wordt in de vorm van lipiden opgeslagen in vetweefsel, om in tijden van vasten te worden aangesproken. Recentelijk is duidelijk geworden dat vetweefsel ook een belangrijk endocrien orgaan is dat een groot aantal adipokines uitscheidt, factoren die onder andere immuunresponsen, bloeddruk, angiogenese, hemostase en reproductieve functies reguleren. Als vetweefsel expandeert, zoals bij overgewicht en obesitas, dan groeit de gemiddelde vetcel, en worden er ook meer vetcellen gevormd uit voorlopercellen. Dit laatste proces wordt adipogenese genoemd. Adipogenese en de biologie van vetcellen worden momenteel intensief bestudeerd, omdat steeds meer duidelijk wordt dat vetweefsel een centrale rol inneemt in de regulatie van ons energie metabolisme en als therapeutisch target voor metabool syndroom. Een veel gebruikt modelsysteem voor adipogenese zijn 3T3-L1 cellen, die in 3 stappen tot volwassen vetcellen kunnen differentieren. In de eerste stap groeien de cellen tot confluentie, daarna ondergaan ze 2 celdelingen onder invloed van een hormooncocktail, om tenslotte de eindfase van differentiatie in te gaan onder invloed van insuline. De middelste fase waarin de cellen tweemaal delen wordt “mitotic clonal expansion” genoemd. Adipogenese wordt gestuurd door een cascade van transcriptiefactoren, met als laatste stap de inductie van de meester-schakelaar PPARγ. Transcriptiefactoren als PPARγ hebben zogenaamde co-regauloreiwitten nodig om de expressie van genen te veranderen. Recentelijk hebben wij aangetoond dat het coregulator eiwit Tip60 essentieel is voor 3T3-L1 adipogenese. In dit proefschrift is de rol van Tip60 in adipogenese verder onderzocht.

In **Hoofdstuk 2** hebben we onderzocht hoe het coregulator eiwit Tip60 gemodificeerd wordt tijdens adipogenese. Voorgaand werk van onze groep had aangetoond dat Tip60 een essentiële rol speelde tijdens adipogenese, en dat expressie van het Tip60 eiwit en niet van zijn mRNA steeg tijdens het differentiatie proces. Omdat dit suggereerde dat het Tip60 eiwit wellicht gestabiliseerd werd tijdens adipogenese, onderzochten wij of Tip60 normaal gesproken afgebroken werd door the ubiquitine-proteasome systeem (UPS) en beschermd werd tegen

afbraak door deubiquitinering tijdens adipogenese. We vonden inderdaad dat expressie van het Tip60 eiwit gereguleerd wordt door polyubiquitinering op meerdere lysine residuen. USP7, een belangrijk deubiquitinerings enzym in 3T3-L1 adipocyten en in muizenvetweefsel, kan Tip60 direct deubiquitineren en daarmee de hoeveelheid Tip60 eiwit vergroten. Remming van USP& expressie en activiteit leidt tot verminderde adipogenese. Onze analyses toonden aan dat met name celcyclus genen onder gemeenschappelijke controle van Tip60 en USP7 staan, en remming van Tip60 en USP7 expressie gaf verminderde MCE. Hieruit concluderen wij dat deubiquitinering van Tip60 een belangrijke rol speelt tijdens de vroege stappen van adipogenese.
Bovenstaand vindingen spoorden ons ertoe aan om ook het ubiquitineringsproces van Tip60 in adipogenese nader te bestuderen (**Hoofdstuk 3**).Een belangrijke stap in eiwit ubiquitinering wordt vervuld door zogenaamde E3 ligases, die ubiquitine aan het substraat zetten en specificiteit aan het systeem verlenen. Van de E3 ligase Mdm2, een enzym dat vooral bekend is vanwege zijn rol in de afbraak van het tumorsuppressor eiwit p53, is aangetoond dat het Tip60 kan ubiquitineren in andere celsystemen, en Mdm2 komt tot expressie in adipocyten. Om deze redenen hebben wij onderzocht of Mdm2 een belangrijke E3 ligase zou kunnen zijn voor Tip60 in (pre)adipocyten. De expressie van Mdm2 verandert weinig tijdens adipogenese. In muizen embryonale fibroblasten die geen Mdm2 bevatten (Mdm2-/- MEFs) bleek Tip60 nog steeds geubiquitineerd te worden. Wij concluderen daarom dat andere E3 ligases waarschijnlijk verantwoordelijk zijn voor Tip60 ubiquitinering en afbraak tijdens adipogenese.
In **Hoofdstuk 4** hebben we de rol van Tip60 in adipogenese en adipocyt functie, en de effecten daarvan op energie metabolisme, onderzocht in een muizenmodel. Omdat homozygote inactivatie van het Tip60 gen resulteert in vroege embryonale dood, hebben we gebruik gemaakt van heterozygote muizen (Tip60+/-). Deze muizen hadden geen ander lichaamsgewicht dan normale muizen, geen afwijkingen in lever en vetweefsel en geen insuline resistentie. Ook wanneer deze dieren werden blootgesteld aan een hoog-calorie dieet bleken ze niet te onderscheiden van normale muizen. Opvallend was dat in Tip60+/- muizen Tip60 mRNA 50% verlaagd was, maar het eiwit onveranderd. De werkelijke rol van Tip60 in adipogenese en adipocyt functie kan daarmee niet onderzocht worden in dit muizenmodel.

In **Hoofdstuk 5** beschrijven we de ontwikkeling van een nieuwe methode om regulatoren van adipogenese te identificeren met behulp van RNAi banken. Screening met RNAi banken wordt bemoeilijkt doordat adipogenese niet homogeen is, en doordat het moment waarop cellen blootgesteld worden aan de RNAi bank zeer kritisch kan zijn, en daarmee de uitkomsten kan bepalen. Wij laten in dit hoofdstuk zien dat onze screening, die gebaseerd is op celdood als uitleesparameter in plaats van differentiatie, in principe kan werken. Deze methode werd gevalideerd door een kleinschalige screening, waarbij UCHL3 werd geïdentificeerd als een belangrijke deubiquitinase in adipogenese.

In **Hoofdstuk 6** hebben we bevindingen van onze studies, beschreven in de voorgaande hoofdstukken, bediscussieerd en geïntegreerd in de huidige literatuur.

Conclusies

De huidige wereldwijde obesitas epidemie, en de daarmee gepaarde aandoeningen als type 2 diabetes en hart- en vaatziekten, vraagt om diepgaande kennis over ons metabolisme en de rol van vetweefsel en vetcellen hierin. Om deze reden wordt er momenteel veel onderzoek verricht naar het complexe samenspel van transcriptionele regulatoren en hun coregulatoren in vetceldifferentiatie en -functie. Soms blijkt de rol van “nieuwe” coregulatoren verrassend complex, zoals hier aangetoond voor Tip60, dat zowel een belangrijke rol in vroege als in late differentiatie lijkt te spelen. Na de identificatie van nieuwe (co)regulatoren is het nodig om hun rol in een heel (model) organisme te bestuderen. Zoals in dit proefschrift aangetoond worden dergelijke studies soms bemoeilijkt door complicaties in het muismodel, en moeten eerst meer geavanceerde modellen (weefselspecifieke knock out, knock-in) ontwikkeld worden. De gecombineerde aanpak van cel-gebaseerde experimenten en geavanceerde muismodellen zal leiden tot een beter begrip van vetcellen in ons metabolisme en zal ons daarmee in staat stellen om nieuwe en beter geneesmiddelen te ontwikkelen.

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Curriculum Vitae

Yuan Gao was born on 19th July 1980 in Benxi, Liaoning Province, China. After finishing her education at Benxi advanced high school, Benxi, China, she started het bachelor studies in Biological Science at Nankai University, Tianjin, China in 1999. After that she moved to The Netherlands to start her master studies, which she performed at the Leiden University Medical Center, Leiden, the Netherlands. She did internships first in the department of endocrinology, under the guidance of Prof. Dr. Ton Maassen, supervised by Dr. Merlijn Bazuine. Then she did internships in the department of pharmacology, under the guidance of Prof. Dr. Ron de Kloet, surpervised by Dr Roel de Rijk. Her thesis topic was entitled “A single nucleotide polymorphism in mineral corticoid receptor”. She then started her PhD training in the Department of Metabolic and Endocrine diseases, UMC Utrecht, The Netherlands, under supervision of Dr. Eric Kalkhoven and Prof. Dr. Ruud Berger. Between early 2006 and mid 2011 she perfomed studies on the role of Tip60 in adipogenesis, which resulted in this thesis.